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(54) Title: FLOW THROUGH ASSAY DEVICE, DIAGNOSTIC KIT COMPRISING SAID ASSAY DEVICE AND USE OF SAID ASSAY DEVICE IN THE DETECTION OF AN ANALYTE PRESENT IN A SAMPLE

(57) Abstract: The present invention relates to an assay device for testing the presence of an analyte in a given sample comprising: a multilayer support whereon a first analyte-binding compound or analyte-binding complex, able to bind said analyte present in said sample, is immobilized, whereby said analyte is able to bind a second enzyme labeled analyte-binding compound or enzyme labeled analyte-binding complex forming a sandwich complex, whereby said sandwich complex is able to generate upon contact with a suitable precipitating substrate for said enzyme-label a colored deposit in a one step procedure. The invention also relates to a diagnostic kit or a method for the detection of an analyte in any medium.

Flow through assay device, diagnostic kit comprising said assay device and use of said assay device in the detection of an analyte present in a sample

Field of the invention

5 The present invention relates to an assay device in particular to a flow through assay device whereon a color deposit is formed upon the presence of an analyte in a test sample. In a second aspect the present invention relates to a diagnostic kit comprising said assay device. In a third aspect the present invention relates to a method for the detection of an analyte present in a sample.

10

Background of the invention

The number of membrane-based rapid immunochromatographic devices on the market is continuing to increase at a very quick pace. Major factors that are contributing to this growth include improvements in conjugate technology and a growing understanding among product
15 developers of the general design principles involved. Although today's immunochromatographic devices come in a wide variety of designs with a diverse assortment of housings, most commonly available tests are based on one of two simple formats.

The most common format is the lateral-flow or dipstick design, which has become familiar
20 through its use in physician-office assays as well as in over-counter tests. A less widespread format is the flow-through or transverse-flow design. Regardless of the format being used, achieving a sensitive and reproducible test requires the manufacturer to have an efficient procedure for applying an analyte-binding compound. Said analyte-binding compound is able to specifically bind the analyte to be analysed. A rapid test is an inexpensive, disposable,
25 membrane-based assay that provides visual evidence of the presence of an analyte in a sample.

By definition, rapid tests provide results in a short time, preferably minutes. It is an object of these tests to be convenient, accurate, reliable, inexpensive, disposable and foolproof. They are preferably also easily and unambiguously to interpret, even by users without much experience.
30 Typically, as little as 200µl of liquid sample is required to perform the test, which is usually complete within 2-5 minutes. No instrumentation is required to perform these tests, which can be used in clinics, laboratories, field locations, and the home – often by inexperienced personnel. The base substrate of a known rapid test is typically a nitrocellulose strip onto which an analyte-binding compound is immobilised, usually an antibody or an antigen. A pad containing dried
35 conjugate is attached to the membrane strip. For the majority of currently available tests, this conjugate pad contains gold particles absorbed with antibodies or antigens specific to the analyte

being detected. When the sample is applied to the device, the liquid sample migrates by capillary diffusion through the conjugate pad, rehydrating the gold conjugate and allowing the interaction of the sample with the conjugate.

5 Gold labels were introduced into membrane-based rapid tests in the late 1980s. Its superior stability, sensitivity and precision make gold suitable for use in rapid test. Nevertheless, high-quality gold conjugate requires the utmost care and attention in order to achieve a final stable and sensitive product. Indeed, many poor-quality, poorly characterized products leads to non-reproducible, non-reliable results. To prevent this, gold colloids have to be evaluated
10 ultrastructurally using a transmission electron microscope (TEM). Such an evaluation should enable the manufacturer to compare the diameter of the colloids to that of calibrated standards and to obtain information about particle sphericity and the overall variance in particle diameter. Evenly shaped with an optimal particle size of 40 nm to 20 nm will allow to set up reliable assays. A mere 5% of odd-shaped particles can influence a test result, making it completely non-reproducible. It is for this reason that the production of such high-quality of gold particles is
15 expensive. This stands in contrast with the above given definition that rapid test should be a low-cost assay.

EP 0 207 152 and US Patent No. 5,958,790 disclose a method for qualitative or semi-quantitative
20 determination of an analyte in a test sample based on the flow-through principle comprising a second analyte-binding compound which becomes immobilized upon contact with the reaction zone whereby a colloidal gold label is being attached to the second analyte-binding compound. A color signal generated by the immobilized colloidal gold form the visual signal. In addition, US Patent No. 5,616,467 defines the optimal size of the particles to be 20 nm in order to increase
25 sensitivity and reproducibility of the test.

However, as discussed above the reproducibility and sensitivity of such gold-based tests is quite low. Further optimalization is needed to increase throughput, sensitivity and reproducibility and decrease costs of these *in vitro* assays.

30 Enzyme immunoassay devices whereby a sandwich complex is produced are known from EP 0 458 231. A flow through device is disclosed whereon a sandwich complex is produced using urease as a label. A cascade of reactions is disclosed in order to produce a colored deposit. A main problem is that said known enzyme immunoassays require several procedural steps such
35 as a cascade of reactions in order to produce a colored deposit. It is an object of the present invention to reduce said multiple step reaction mechanism.

EP 0 125 118 discloses an immunoassay whereby enzyme-linked sandwiches are formed on a dipstick surface and whereby an insoluble deposit is formed. Several wash steps are required in obtaining said colored deposit.

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US Patent No. 5,958,790 discloses a method for qualitative or semi-qualitative determination of an analyte in a test sample based on a flow-through principle whereby a visual signal is generated by the immobilized colloidal gold particles.

10 Object of the present invention

The aim of the present invention is thus to provide a cheap, easy, fast, reproducible, sensitive test which is reliable for the detection of analytes in a sample.

The present invention uses conjugates or analyte-binding compounds labeled with enzymes able
15 to form insoluble precipitates when (an) analyte(s) is (are) present in a test solution in a flow-through set up. An object of the present invention is directed towards a further optimization of the rapid-test concept through a balanced consideration of each parameter resulting in a reproducible and sensitive test. A further object of the present invention is directed towards obtaining a cheap assay device which can be used in high throughput set up and which can be
20 easily used at home or in field locations.

A main object of the present invention is to provide a reliable one-step procedure, in order to obtain a colored deposit, integrated in the flow-through assay device. The use of such a one-step procedure allows to generate a faster, easier, cheaper test system, but also to improve the
25 reliability and reproducibility of the corresponding test. Indeed, by omitting procedural steps, errors, such as pipetting or handling errors, may become limited or even negligible. The use of a one-step detection procedure, as proposed in present invention, in contrast to the use of a multiple step reaction detection mechanism as described in EP 0 458 231 results in this effect. In addition, in the device/method/kit of present invention washing steps are omitted. Such washing
30 steps are essential for the sensitivity/reproducibility for most prior art tests, e.g. EP 0 458 231 and EP 0 125 118. Besides the improvement of above-mentioned features, the device/method/kit of present invention also allows a permanent record of the results which is not the case when using gold particles. A permanent record allows the person skilled in the art to use obtained results with results obtained from previous/later experiments. This makes comparison of inter-assay-results
35 easier and more reliable. In addition, the production of a kit according to US Patent No.

5,958,790 requires more labour and is more expensive and corresponding test results are less reliable.

As a general conclusion, the present invention describes a faster, easier, cheaper device/method/kit which is more comparable, reliable and reproducible than already known test devices/methods/kits.

These aims separately, partly combined or all together have been met by the present invention.

Summary of the invention

10 According to the present invention an assay device is provided for testing the presence of an analyte in a given sample comprising: a multilayer support whereon a first analyte-binding compound or analyte-binding complex, able to bind said analyte present in said sample, is immobilized, whereby said analyte is able to bind a second enzyme labeled analyte-binding compound or enzyme labeled analyte-binding complex forming a sandwich complex, whereby
15 said sandwich complex is able to generate upon contact with a suitable precipitating substrate for said enzyme-label a colored deposit in a one step procedure.

In the present invention a 'complex' is to be understood such that the analyte-binding compound may form part of a cluster of molecules, whereby one or more compounds present in this cluster
20 may bind the analyte separately or cooperatively. Alternatively, the analyte may also form part of complex comprising one or more which might be identical or different analytes. The other members in this cluster or complex may be peptides, proteins, lipids, nucleic acids or organic molecules.

25 By combining the flow through system with the use of a specific precipitating substrate the inventors found surprisingly that highly reliable, fast, sensitive and high-throughput test conditions could be created for the detection of analytes in a test sample, due to the fact that a color deposit is directly obtained in merely one step. In addition, the production of these assay devices are easy and cheap and does not demand extra high-quality of products.

30 Another object of the present invention is directed towards a further optimization of the rapid-test concept through a balanced consideration of each parameter resulting in a reproducible and sensitive test such as composition of the membrane, pore size of the membrane, optimization of the labeled reagent system to detect the analyte, composition of the coating buffer, choice of the
35 analyte-binding compound, application procedure, new blocking procedure (post-coating by immersion during a couple of hours has not been described before), storage and stability of the

membrane, stability of the result, costs. Through this optimization procedure all washing steps which were previously needed to clear the background signals can be omitted which makes that the test is not only cheaper but also easier and faster than the test systems described until now.

5 The present flow-through set up allows a "high throughput" approach which may be necessary when a high number of samples needs to be analyzed. The described invention also allows reproducible testing of test samples which may be essential when used for clinical diagnosis. The inventors found that the sensitivity of the assay using the device as described by the invention is comparable with the most optimized condition as described for the gold-based tests. These
10 devices can be used for a wide range of applications not only for clinical applications but also for agricultural, environmental and veterinary applications. Such tests can be performed outside the laboratory without laboratory equipment by physicians, laboratory technicians or less trained personnel.

15 In summary, the present invention relates to an assay device complying with the requirements which are essential for the acceptance of such tests on the market: ease of use, small sample volumes, speed, i.e. within 3-5 minutes, reliability and low sales price. Moreover, these tests also feature the same technical performances, i.e. high sensitivity and specificity, long-term stability as those provided by instrumented testing in laboratory.

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According to the present invention, said multilayer support of said assay device of the invention as described above comprises:

- a) an upper cover layer of a water-impermeable material having at least one hole, whereby said hole at least partly exposing a test zone,
- 25 b) an intermediate porous layer comprising at least one insoluble porous material whereon the first analyte binding compound is able to bind in said test zone, and
- c) a lower absorbent layer comprising at least one layer of a hydrophilic material.

According to the present invention the water-impermeable material of said upper cover layer as
30 defined above is chosen from plastic adapted to the sample to be tested comprising polypropylene, polyvinylchloride or styrene-ethylene/butylene styrene (SEBS) (Rubin (1990), Schouten and van der Vegt (1987)). This prevents unwanted background signals caused by hydrophobic or hydrophilic interaction of some of the components present in a sample with the plastic.

35

The assay device of the present invention comprises at least one hole in the water-impermeable material having a diameter of at least 1 mm overlaying a test zone. The hole limits the surface of the porous membrane to which the sample is exposed to and, when a large volumes are spotted onto this multilayer device, the hole also helps the liquid to be absorbed by the area defined by the hole. The hole is not limited by the size of the test zone. Both hole and test zone may have any form such as a circle, square, triangle, cross or any regular or irregular surface.

The test zone is part of the porous layer where the first-analyte-binding compound or capturing molecule has been spotted. The test zone may be between 1 to 10 mm wide. A test zone with a size smaller than 1 mm is possible but in such case interpretation, i.e. reading of results may be difficult. A test zone with a size higher than 10 mm is possible but in such case, more volume of reagents is needed. Nevertheless, in some cases the use of a large test zone is crucial, e.g. for the analysis of food samples. The soaking of larger sample volume, e.g. from 0,1 ml to 2 mls may allow concentration onto the membrane of the analyte to be detected. Said test zone may have a diameter of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 mm. Preferentially said test zone has a diameter of 3 to 4 mm. For such a zone sample volumes between 5 to 500 μ l are preferentially used. The volume of the sample added is preferentially equal or smaller than the volume of the reagent solution. E.g. if a 15 μ l sample is applied, a 25 μ l of the reagent solution is used; when a 25-30 μ l of sample is used, 50 μ l of reagent solution is applied. If the test zone is larger than 4 mm or if sample volumes are larger than 100 μ l the composition of diluent buffer may be modified by the addition of sucrose going from 1% up to 40%. Possible concentrations are 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39 and 40%. Sucrose may be added into the diluent buffer in order to slow down the flow rate phenomenon in order to get a lower detection limit and to avoid the appearance of a heterogeneous colored spot.

The assay device of the present invention comprises, preferably an intermediate insoluble porous layer to which an analyte-binding compound or to which a capturing molecule is bound. This layer is capable of filtering complex suspensions, e.g. cellular material from a sample being assayed if the analyte is associated with the cellular material. In the last case, the membrane or filter is selected to have a pore size which permits this separation. Any of a variety of filtering members may be used including glass fiber filters and filters of various synthetic or natural materials. According to present invention, the intermediate insoluble porous material is chosen from a group comprising nylon, nitrocellulose, cellulose, fiberglass, polysulfone, polyvinylidene difluoride, polyester or any other polymeric material to which biological substances may bind. Preferentially, the intermediate insoluble porous material is nitrocellulose (Advanced

Microdevices (PVT) LTD. 21, Industrial Area, Ambala Cantt, 133 001 India). In addition, the present invention suggests that the intermediate insoluble porous material has pores with a diameter between 0.1 and 12 μm and has a thickness up to 2500 μm . Membranes with pore sizes of 0.1, 0.2, 0.45, 0.8, 1.2, 3.0, 5.0 8.0 and 12 μm may be used. Membranes with pore sizes
5 of 0.45, 0.8, 1.2 μm are the most relevant; membranes with pores of 0.45 μm and a thickness of 500 μm are preferentially used. The thickness of the membrane seems to influence the flow rate and the quality of the results.

All of these embodiments of the present invention are based on experimental evidence as
10 described in the example section.

In the present invention the Intermediate Insoluble porous membrane is a layer such as membrane or filter to which a maximum of first-analyte-binding-compound or capturing molecule can be bound. In some cases, it may be necessary not to load a maximum of this first analyte
15 blinding compound or capturing molecule onto this surface. The term "bound" in the present invention is intended to embrace any means for fixing the first analyte-binding compound or capturing molecule to the porous member. In addition, this term includes all means that can be used for setting up covalent and non-covalent binding. The material of the porous member is selected from a material to which the analyte-binding compound or, if used, capturing molecule
20 can be bound. The invention does not exclude the possibility to bind the analyte immediately onto this porous surface.

In case covalent linkage is chosen and the molecules to be bound are proteineous, e.g., antibodies or antigens, the porous membrane has amino group residues or into which such
25 groups have been introduced by chemical means. Amino groups permit a protein to be coupled to it by the well known glutaraldehyde method. Alternatively, antibodies can be coupled to glass fibers through aminosilanes. Other natural or synthetic materials which can be coupled directly or through intermediates to an analyte-binding compound may also be used.

Although covalent linkage of the first analyte-binding compound or the capturing molecule might
30 guarantee more stable situation of the assay, it is shown by the present invention that the use of non-covalent linkages in a flow-through setup also results in reliable assay conditions. As non-covalent linkages are based on charge or hydrophobic interaction, it is more easy and less laborious to perform such linkages. Therefore, a preferred embodiment of present invention is to
35 coat the first analyte-binding compound or the capturing molecule non-covalently on said intermediate insoluble porous member of the assay device.

The coating of the porous layer with the capturing or first-analyte-binding compound is performed preferentially via immersion of the layer in coating and post coating solutions followed by a drying procedure. However, alternatively a spraying or a spotting process can be used for the binding of
5 molecules with high affinity.

Preferably the antibody preparation comprises a monoclonal antibody even though polyclonal antibodies from antisera may be used. In this respect also crude antibody preparations may be used for this purpose. Techniques for polyclonal and monoclonal antibody preparation and/or
10 purification are now well known and require no citation here.

In yet another embodiment of the invention, the porous member of the device of the present invention may have capturing molecules bound to it. As used herein, the term "capturing molecule" is intended to refer to agents which will bind selectively to the first analyte-binding
15 compound. The use of a capturing molecule bound to the porous member makes it possible to simplify development and preparation of the porous member useful in ligand-receptor assays. Here "ligand" is defined as being the analyte, "receptor" can be defined as being the analyte-binding compound. For example, if an analyte-binding compound is bound to the porous member, it may be necessary to modify the binding procedure in order to optimize the binding of
20 each analyte-binding compound required for a panel of assays. However, a single capturing molecule or a mixture of multiples capturing molecules or a mixture multiples analyte-binding compounds such as antigens or antibodies bound to the porous member may be employed in a plurality of assays. As a result, the development effort and manufacturing procedures may be greatly simplified when such a "universal" porous member is possible. Consequently, depending
25 on the strategy used, the coating of the first analyte-binding compound may be situated before or after the assembly of the assay device and may be carried out by the manufacturer or by the person who performs the assay. In addition, assembly of the device itself can also be performed by the person who carries out the assay.

30 The lower layer of the device of the present invention is an absorbent member or layer having capillary passage ways generally transverse to the upper and lower surfaces. The lower absorbent layer is assembled with the intermediate porous layer in a manner which permits direct communication between the pores or interstices of the porous layer and the capillaries of the absorbent layer. Thus, as a liquid is applied onto the intermediate porous layer and is
35 subsequently absorbed by the lower absorbent layer and saturates it, the liquid is drawn through capillary force into the absorbent member. As a result, flow can be induced through the lower

absorbent layer when a liquid sample is applied to the surface of the intermediate porous layer even though the hydrostatic pressure of the fluid is so low that unaided it could not flow through the intermediate layer without the application of pressure to force it through or a vacuum to draw it through. The absorbent layer comprises at least one layer of hydrophilic material in contact with
5 and positioned on the side of the insoluble porous layer opposite the side of the cover layer.

The selection of material for absorbent layer is not critical and a variety of fibrous filter materials can be used. A useful material is cellulose acetate fibers oriented as in a cigarette filter. Those skilled in the art will appreciate that other absorbent members made of polyester, polyolefin or
10 other materials may be used in place of cellulose acetate. Present invention suggests that the hydrophilic material of the device allows communication between the porous material and the absorbent layer and is preferentially AP120, provided by the company mdi (Advanced Microdevices (PVT) LTD. 21, Industrial Area, Ambala Cantt, 133 001 India). Alternatively, equivalent filter pads may be used. These absorbent layers or filter pads can be provided by
15 several companies involved in membrane technology, e.g. Schleicher & Schuell, Sartorius and Millipore.

As already mentioned above, according to present invention said first and the second analyte-binding compounds are substances which specifically bind the analyte and are chosen from a
20 group comprising peptides, proteins, lipids, nucleic acids and organic molecules. When the porous member has a first analyte-binding compound bound to it, the analyte-binding compound is selected for its ability to selectively bind directly with the analyte. For example, if the analyte is an antigen, the analyte-binding compound may be an antibody, monoclonal or polyclonal which specifically binds the analyte, preferably a monoclonal antibody. If the analyte is an antibody, the
25 analyte-binding compound may be an antigen or anti-antibody. If the analyte is an enzyme, the analyte-binding compound may be a receptor or a substrate for the enzyme. If the analyte is a nucleic acid, for example, RNA or DNA, the receptor may be a complementary oligomer of DNA or RNA or a nucleic acid binding protein.

30 Preferentially, said first analyte-binding compound and/or said second analyte-binding compound are an antibody which binds specifically the analyte. In this respect, present invention also defines said antibody being preferentially a monoclonal or polyclonal or an antibody preparation thereof. The term "specific binding" implies that there is substantially no cross-reaction of the antibody with other proteins. The term "antibody preparation" covers any solution containing
35 antibodies such as serum or solutions containing any antibody derivative. The antibodies according to the invention may be produced according to techniques which are known to those

skilled in the art. Monoclonal antibodies may be prepared using conventional hybridoma technology as described by Kohler and Milstein (1979) (Kohler, F. and Milstein, C. 1995. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 256: 495-497.). Polyclonal antibodies may also be prepared using conventional technology well known to those skilled in the art, and which comprises inoculating a host animal, such as mouse, with a protein or epitope according to the invention and recovering the immune serum. The present invention also includes fragments of whole antibodies which maintain their binding activity, such as for example Fv, F(ab') and F(ab')₂ fragments as well as single chain antibodies.

10 The present invention further discusses that the first analyte-binding compound might be directly or indirectly coupled to the porous layer of the device. When indirectly coupled, the device is first coated with a capturing molecule as described above which specifically binds the first analyte-binding compound followed by the coating of the first analyte-binding compound. For example, if the analyte is an antigen and the first analyte-binding compound is an antibody, for example, a mouse IgG antibody, preferably a monoclonal antibody, the capturing may be an antibody, preferably a monoclonal antibody, against murine IgG. In other cases the first analyte-binding compound may be conjugated with a moiety which binds selectively with the capturing molecule. For example, the moiety may be a hapten and the capturing molecule an antibody against said hapten. A possible hapten is fluorescein. In other cases, the capturing molecule may be avidin or streptavidin. In such case, the first analyte-binding compound will have biotin bound to it. In other cases, the first analyte-binding compound may be nucleic acid oligomer, or have such an oligomer bound to it, and the capturing molecule may be a nucleic acid segment complementary to a portion of the first analyte-binding oligomer which does not impair binding with the analyte. Those skilled in the art will appreciate from the foregoing that a variety of capturing-molecule/first-analyte-binding compound combinations may be employed.

According to the present invention the second analyte-binding compound is labeled with an enzyme which upon interaction with a precipitating substrate results in one step in a colored deposit. It is the use of such a precipitating substrate in such a defined assay device as described by the invention that makes the analysis of the analyte within a test sample cheap, easy, stable and reliable. This second analyte-binding compound can also be referred to as "conjugate".

In analogy with the use of a capturing molecules as described above, the present invention also relates to a diagnostic assay device wherein the analyte is detected indirectly by the use of a detection molecule. Indeed, it is possible that said second analyte-binding compound is further

bound by a detection molecule labeled with a different enzyme (E2) than the enzyme present on the second analyte-binding compound (E1), whereby this E2 enzyme upon interaction with a precipitating substrate using a one step procedure results in a colored deposit. In this way, amplification of the signal can be obtained.

5

Alternatively, said second analyte-binding compound is not enzyme-labeled and is further bound by a detection molecule labeled with an enzyme which upon interaction with a precipitating substrate using a one step procedure results in a colored deposit.

10 Indirectly coupling of the first-analyte-binding compound or the indirect detection of the second-analyte binding compound can be realized via an avidin/biotin, streptavidin/biotin antibody/antigen, antibody/hapten, receptor/ligand, sugar/lectin, complementary nucleic acid, i.e. RNA or DNA, or combination thereof, enzyme/substrate, enzyme/cofactor, enzyme/inhibitor or immunoglobulin/ Staphylococcal proteinA interaction.

15

According to the present invention, said test sample can be chosen from a group comprising cell fractions, serum, whole blood, urine, plasma i.e. for human or animal diagnostic testing; soil, mud, minerals, water, air i.e. for environmental testing; any food materials i.e. for food testing; or any other medium/suspension/hard material which can be used for one of these purposes. The medium analysed can be both solid or liquid in nature. It is evident when solid materials are used, these are first dissolved in a suitable solution. According to the invention, this solution is not always a real "buffer" with at least 2 well balanced components. It may be a strong hypotonic solution such as NaCl alone or an extraction solution such as with alcohol.

20

25 According to the present invention, said test sample can be applied undiluted or in a diluted form using a diluent buffer and for which the dilution factor is adapted to the analyte to be detected (claim 23). Said dilution may vary from $\frac{1}{2}$ up to 100.000. Dilutions of $\frac{1}{2}$, $\frac{1}{3}$, $\frac{1}{4}$, $\frac{1}{5}$, $\frac{1}{6}$, $\frac{1}{7}$, $\frac{1}{8}$, $\frac{1}{9}$, $\frac{1}{10}$, $\frac{1}{15}$, $\frac{1}{20}$, $\frac{1}{25}$, $\frac{1}{30}$, $\frac{1}{35}$, $\frac{1}{40}$, $\frac{1}{45}$, $\frac{1}{50}$, $\frac{1}{55}$, $\frac{1}{60}$, $\frac{1}{65}$, $\frac{1}{70}$, $\frac{1}{75}$, $\frac{1}{80}$, $\frac{1}{85}$, $\frac{1}{90}$, $\frac{1}{95}$, $\frac{1}{100}$, $\frac{1}{150}$, $\frac{1}{200}$, $\frac{1}{250}$, $\frac{1}{300}$, $\frac{1}{350}$, $\frac{1}{400}$, $\frac{1}{450}$, $\frac{1}{500}$, $\frac{1}{550}$, $\frac{1}{600}$, $\frac{1}{650}$,
 30 $\frac{1}{700}$, $\frac{1}{750}$, $\frac{1}{800}$, $\frac{1}{850}$, $\frac{1}{900}$, $\frac{1}{950}$, $\frac{1}{1000}$, $\frac{1}{1250}$, $\frac{1}{1500}$, $\frac{1}{1750}$, $\frac{1}{2000}$, $\frac{1}{2250}$, $\frac{1}{2500}$, $\frac{1}{2750}$, $\frac{1}{3000}$, $\frac{1}{3250}$, $\frac{1}{3500}$, $\frac{1}{3750}$, $\frac{1}{4000}$, $\frac{1}{4250}$, $\frac{1}{4500}$, $\frac{1}{4750}$, $\frac{1}{5000}$, $\frac{1}{5250}$, $\frac{1}{5500}$, $\frac{1}{5750}$, $\frac{1}{6000}$, $\frac{1}{6250}$, $\frac{1}{6500}$, $\frac{1}{6750}$, $\frac{1}{7000}$, $\frac{1}{7250}$, $\frac{1}{7500}$, $\frac{1}{7750}$, $\frac{1}{8000}$, $\frac{1}{8250}$, $\frac{1}{8500}$, $\frac{1}{8750}$, $\frac{1}{9000}$, $\frac{1}{9250}$, $\frac{1}{9500}$, $\frac{1}{9750}$, $\frac{1}{10.000}$, $\frac{1}{11250}$, $\frac{1}{11500}$, $\frac{1}{11750}$, $\frac{1}{12000}$, $\frac{1}{12250}$, $\frac{1}{12500}$, $\frac{1}{12750}$, $\frac{1}{13000}$, $\frac{1}{13250}$, $\frac{1}{13500}$, $\frac{1}{13750}$, $\frac{1}{14000}$,
 35 $\frac{1}{14250}$, $\frac{1}{14500}$, $\frac{1}{14750}$, $\frac{1}{15000}$, $\frac{1}{15250}$, $\frac{1}{15500}$, $\frac{1}{15750}$, $\frac{1}{16000}$, $\frac{1}{16250}$, $\frac{1}{16500}$, $\frac{1}{16750}$, $\frac{1}{17000}$, $\frac{1}{17250}$, $\frac{1}{17500}$, $\frac{1}{17750}$, $\frac{1}{18000}$, $\frac{1}{18250}$, $\frac{1}{18500}$, $\frac{1}{18750}$, $\frac{1}{19000}$,

1/19250, 1/19500, 1/19750, 1/20.000, 1/30.000, 1/40.000, 1/50.000, 1/60.000, 1/70.000, 1/80.000, 1/90.000 and 1/100.000 are possible. The dilution used depends on which sample is used. For example, for serological applications, i.e. samples coming from urine, serum, plasma or cell fractions the dilution range may be from undiluted to 1/200; for microbiological applications, e.g. detection of microbiological contamination in food and water the range of dilutions may be extended to 1/10.000. It is evident that for samples carrying high amounts of the analyte or when using an analyte-binding compound which has a high affinity for the analyte, dilutions up to 100.000 may be performed.

- 10 Said diluent buffer has the function to dilute the sample but in certain cases also the effect on the improved presentation of the analyte to be recognized by the analyte-binding compound. Therefore a solubilizing agent such as EDTA or SDS may be included.

In addition, according the analyte(s) to be detected, the composition of the diluent buffer of the sample(s) needs to be adapted for the detection of the analyte. For example, if the sample to be tested is isolated from food, a sonication process may be required in order to dissolve aggregates; if the sample to be tested is isolated from urine, adjustment of pH may be needed; if the sample contains lipids i.e. "fat sample", delipidation may be requested. An hemolytic solution may be requested in order to hemolyse the red blood cells. In some cases the salinity needs to be adapted. All measures listed may be necessary to prevent clogging of the system or to optimize the condition wherein the analyte needs to bind the analyte-binding compounds. Other examples of such adaptations are for human application: a sample from synovial fluid for the detection of rheumatoid factors has to be treated with hyaluronidase. For microbiological application: a sample from food for the detection of contaminants have sometimes to be enriched through the addition of some nutriment or lysed by the addition of lytic agents into the diluent buffer. The diluent buffer may also comprise a preservative such as thimerosal or sodium azide.

According to the present invention, said analyte is a compound abnormally or normally present or absent in the sample. So, the devices according to the present invention can be used to detect the absence or presence of an analyte in a sample; nevertheless, they can also be applied to evaluate the quantity of a certain analyte into a medium whereby a decrease or an increase in analyte-concentration can be studied. If the device according to the invention is used to diagnose a disease then the analyte is a cellular compound either of intracellular, membrane or extracellular origin. In this case, the term "abnormally" implies that the presence *per se*, an increase or a decrease of the present level or the absence of the analyte is indicative for a disease. Examples of analytes which are "normally present" are LH and TSH; these are normally

present and may abnormally increase or decrease in certain diseases. According to present invention, said compound is selected from the group comprising antigens and antibodies; whereby said antigen may be chosen from the group comprising any biological agent such as bacteria, viruses, molds, mycobacteria, parasites, pathogens; or molecules such as peptides,
5 proteins, lipids, organic molecules, nucleic acid oligomers.

According to the present invention, said antigen may be chosen from the group comprising proteins for which the level abnormally increases in certain diseased states or abnormally increases in food material.

10

According to the present invention said antigen may be a bacterium or a toxin produced by a bacterium in food material or in biological fluids. Examples are: *Salmonella sp.*, *E.coli sp.*, *Listeria sp.*, *Clostridium sp.*, *Staphylococcus sp.*, *Campylobacter sp.*, *Mycobacterium*, *Streptococcus*...; i.e. urine, blood, serum, feces, cerebral fluid. Such bacteria are normally absent in safe food or in
15 healthy people.

More specifically, the present invention defines that said antigen may be chosen from the group comprising C-reactive protein (CRP), troponin, myoglobin, HCG (human chorionic gonadotrophin, LH (Luteinizing hormone), rheumatoid factors, cardiolipin, centromere (kinetochore proteins),
20 histones, Jo-1 (eponymously named, same as histidyl tRNA transferase), lupus coagulant, myeloperoxidase, nucleolar auto-antigens, e.a. PM-Scl = polymyositis-Scleroderma, RNP (ribonucleoproteins) (eg. U1RNP), Scl70 (same as topoisomerase 1), Sm (eponymously named as Smith antigen, same as nuclear antigen), SSA/Ro (Sjögren syndrome antigen), SSB/La (Sjögren syndrome antigen, thyroglobulin, cell surface lipoproteins, Thyroid auto-antigens,
25 collagen, ANCA (anti-neutrophil cytoplasmic antibodies) (claim 29). CRP is increased in case of infections, myoglobin in traumatic states, troponin in case of myocardial infarction, rheumatoid factors in case of inflammatory states and HCG in case of pregnancy. Additionally, said protein may be chosen from the group comprising a beta-Adrenoreceptor, TSH-receptor, Insulin receptor, Acetylcholine receptor, Gastrin receptor, pyruvate dehydrogenase. Nevertheless, this
30 assays device can be used for the detection of many other substances and is not limited to the listed examples.

The assay device of the invention can also be used to detect an enzyme by binding a compound carrying a high affinity for this enzyme to the porous member as the assay receptor. This
35 compound might be the substrate of the enzyme or any other protein or molecule that binds specifically to this enzyme. A labeled antibody against the enzyme can be used to detect

formation of a receptor-enzyme complex on the porous member. As known by the skilled person in the art also other compounds which have a specifically binding capacity towards this enzyme can be used.

- 5 These proteins are recognizable by analyte-binding compounds such as antibodies. Positive controls preferentially contain purified proteins from natural sources; nevertheless, recombinant proteins with a similar folding as found in the natural protein may be used. Consequently, the analyte-binding compound needs to preferentially recognize an analyte with a conformation as found in the natural protein; nevertheless, also analyte-binding compounds recognizing both
10 correctly folded protein and denatured protein might be used.

- According to the present invention the analyte might comprise an antibody belonging to any class of immunoglobulins such as IgE, IgG, IgM, IgA, IgD. The presence of a specific antibody in a sample might give indications on stage, location and nature of a disease. An increase of IgE is a
15 measure for allergic reactions and presence of helminthic parasites; the increase of IgG confirms the presence of infections which is already in an extended stage; IgM indicates that infections are present in an early state. IgA can be especially detected in secretions and IgD are present on membranes of B cells. Present invention illustrates the detection of anti-mitochondrial antibody type M2 and rheumatoid factors antibodies by the FT method of present invention. However, the
20 FT method of present invention can be used to detect other antibodies, even to detect antibodies from a different Ig class or antibodies which are present on cell surfaces. The examples and figure legends serve to illustrate the invention and are by no way to be understood as limiting the present invention.

- 25 The assay device of this invention may also be used in assays for an antibody which employ an antigen as first analyte-binding compound on the solid phase and which use labeled antigen or labeled anti-antibody as the second analyte-binding compound. The latter is particularly suited to allergy specific assays where the first analyte-binding compound is an allergen bound to the porous member and the second analyte-binding compound is an antibody, preferably a
30 monoclonal antibody to IgE. In other cases, the IgG response to allergens may be measured similarly, i.e., by using an antibody, such as a monoclonal antibody against IgG, as the second analyte-binding compound. Other antibody tests which can be carried out in this manner include tests for detection of viral infections, e.g. herpes, rubella, hepatitis, cytomegalovirus, rotavirus, RSV, HTLV-III, HIV or bacterial infections, e.g. *Streptococcus A&B*, *Chlamydia*, *M.tuberculosis*,
35 *M.pneumoniae*, *H.pylori*, *Clostridium*, *E. coli*, *Listeria*, *Staphylococcus*, *Clostridium*.

Additionally, the present invention defines that said nucleic acid oligomer may be chosen from the group comprising DNA, RNA, DNA/RNA hybrid or chemically analogues thereof, genetically modified or not. With DNA or RNA molecules is meant complementary DNA (cDNA), genomic DNA (gDNA), double stranded-DNA (dsDNA), single stranded-DNA (ssDNA), nuclear RNA (nRNA), transfer RNA (tRNA), messenger RNA (mRNA) and ribosomal RNA (rRNA). RNA molecules may also include dsRNA. When nucleic acid oligomers are used which are not isolated from any living organisms, also RNA/DNA hybrid molecules or oligomers consisting of non-natural nucleotides such as inosines might be used. In these cases, the porous member can be coated with a nucleic acid oligomer as a first-analyte-binding compound for the detection of nucleic acid material in a sample. The first-analyte-binding compound may be an oligomer of DNA, for example, complementary to a sequence in the nucleic acid of interest and can be used to bind either RNA or DNA. Subsequently, the detection of the formed complex can be done using a second nucleic acid oligomer complementary to a non-interfering region of the nucleic acid ligand of interest, the second oligomer being labeled to permit detection.

According to present invention, the enzyme-label, which is coupled to said second analyte-binding compound or said detection molecule, reacts in one step with a precipitating substrate and may be chosen from the group comprising horse radish peroxidase (HRP), alkaline phosphatase (AP), and dehydrogenase. Dehydrogenase can be specified as being for instance glucose-6-phosphate dehydrogenase, lactate dehydrogenase or a malate dehydrogenase. For the enzymes glucose oxidase, cholesterol oxidase, urease, β -galactosidase, and lysozyme no precipitating substrates have been defined so far. Nevertheless, the principle for using these in the same context is similar as described for the above mentioned substrates.

In the present invention said enzyme-label is covalently or non-covalently bound to the second analyte-binding compound. For linkage, the second-analyte binding compound should have a reactive group that is complementary to a reactive group on the enzyme-label. For instance, a free carboxyl group of the second-analyte binding protein complements the amino terminus of enzyme-label such that an amide bond can be made. Alternatively, the analyte-binding compound should be capable of modification to have a reactive group that complements a reactive group of the enzyme label. The second analyte-binding compound can be linked to the linking moiety directly or via a spacer moiety. Those of skill in the art will recognize that, while in most instances the analyte-binding compound and enzyme-label will be linked directly, in some instances it may be desirable to space the linking moiety away from either or both parts with a spacing moiety. It will be recognized that virtually any linkage that is stable to the conditions of use and that can be readily formed without denaturing or otherwise degrading the analyte-binding

compound and/or enzyme-label may be employed. Thus, the analyte-binding compound and enzyme-label may include virtually any reactive group that is complementary to, i.e. able to covalently react with, the respective terminus of the linking moiety to which it will be attached may be utilized. Suitable groups complementary to the linking moiety amino terminus include, for example, carboxy groups, esters including activated esters such as NHS-esters, acyl azides, acyl halides, acyl nitriles, aldehydes, alkyl sulfonyl halides, halotriazines, imidoesters, isocyanates, isothiocyanates, sulfonate esters, etc. Suitable reactive groups complementary to the linking moiety carboxy terminus include, for example, amines, alcohols, alkyl halides, thiols, hydrazines, diazoalkanes, sulfonate esters, etc. Conditions for forming covalent linkages between a plethora of complementary reactive group pairs are well known. Preferably, each linkage between the linking moiety and the biologically active agent and the masking moiety is an amide. Conditions for linking molecules together having complementary amino and carboxy groups to form amide linkages are well-known (see, e.g., Merrifield 1997 Merrifield, B. 1997. Concept and early development of solid-phase peptide synthesis. Methods Enzymol. 289: 3-13.). Examples of non-covalently linkages were already illustrated above.

According to a preferred embodiment of the present invention, said substrate of HRP may be chosen from the group comprising TMB (tetramethylbenzidine) and AEC (3-amino-9 ethylcarbazole). When using the horse radish peroxidase, the enzymatic reaction consists of the reaction of 3-3',5-5' tetramethylbenzidine with the enzyme and hydrogen peroxidase forming in one step insoluble blue free radical cation 1-electron oxidation product.

The mentioned substrates are commercially available. Nevertheless, the inventors illustrate in the present invention that the source of the TMB solution may exert an influence on the quality of the obtained results. Also other reagents known in the art may form suitable substrates. AEC produces a red end product that is soluble in alcohol. This precipitable substrate is used mainly in immunoblotting and immunohistochemical staining techniques, cf. histology.

According to a preferred embodiment of the present invention for alkaline phosphatase said substrate may be chosen from the group comprising BCIP (5-bromo-4-chloro-3-indolylphosphate) and BCIP-NBT(5-bromo-4-chloro-3-indolylphosphate-nitrobluetetrazolium). The mentioned substrates are commercially available. Other reagents known in the art are also suitable. When BCIP reacts with alkaline phosphatase, the free halogenated indoxyl derivative dimerizes, forming an insoluble indigo dye. This product is commonly used for immunoblotting and immunohistochemical staining techniques, cf. histology. The BCIP-NBT system is based on the hydrolysis of BCIP and reduction of NBT producing a deep purple reaction product. This reagent

may be used for immunoblotting, immunohistochemical staining techniques and *in situ* hybridisation techniques.

For a dehydrogenase, the substrate to be used according to the present invention may be NBT
5 (nitrobluetetrazolium). Nitrobluetetrazolium is also used in analyte detection systems that employ dehydrogenase activity. The mentioned substrate is commercially available. Other precipitable substrates known in the art may be used as well.

Subsequently to the formation of the precipitation, the TMB precipitate may be fixed using a
10 reagent comprising polyvinyl alcohol supplemented with dioctyl sulfosuccinate and dimethyl formamide. Such solution stops the enzymatic reaction and allows permanent record of the result.

The assay device according to the present invention may be used for qualitative (yes/no answer);
15 semi-quantitative (-/+ /++ /+++ /++++) or quantitative answer. In a preferred embodiment of the invention, the assay device contains at least 1 test zone which may be used for standard(s) and/or positive and/or negative and/or cut-off control(s). With standard is meant a calibrator. As calibrators, a set of at least one analyte solution with known concentration might be used. This helps to evaluate the analyte concentration into a sample quantitatively or semi-quantitatively.
20 The use of a positive built-up control does show if the test works. The use of a built-up cut-off may be used for the qualitative interpretation of results (yes/no answer) control but no quantitative information can be retrieved from it. For specific tests, it might be advisable that more than one test zone is present in the assay device of the invention. For example when using it to test allergy, 1 card with 6 holes for each panel of allergens can be developed. Alternatively,
25 to test the cardiac markers, e.g. troponin, myoglobin, a card whereon the several cardiac markers are tested in the same time would be optimal. Said test zone(s) can be located within the same hole of the upper cover layer or in separate holes.

The present invention also relates to the interpretation of the observed signal using a card
30 system or using a reader. A "card system" can be defined as being a holder where colored spots of different color intensities are indicated. When the test is reproducible the visualized color can be linked to a concentration of the analyte into the tested sample. This holder might comprise holes within these color spots. In this way the color of the holder can be easily compared to the color formed in the assay device by overlapping both devices. Alternatively, a reader measuring
35 the reflectance can be used to monitor color deposits. Any reader known by the skilled person in the art measuring reflectance can be used. In the examples the VISI-CHROMA™ VC-100 of

Biophotonics S.A. (Lessines, Belgium) is used; nevertheless, other systems such as reflectance readers may be used, for example Nycomed readers.

5 The VISI-CHROMA reader which is used by the inventors for the interpretation of results uses a color CCD camera (charge coupled device) allowing an accurate measurement in a tri-stimulus way (3 filters) on a selected area of the membrane. Such technique allows a very accurate measurement of the colored deposit (dot).

The present invention also relates to a diagnostic kit comprising:

- 10 - an assay device as defined in the present invention provided with a first analyte-binding compound or analyte-binding complex,
- a second solution comprising an enzyme-labeled second analyte-binding compound, an enzyme-labeled second analyte-binding complex or an enzyme-labeled detection molecule,
- 15 - a third solution comprising a precipitating substrate for the enzyme linked to the second analyte-binding compound, an enzyme-labeled second analyte-binding complex or an enzyme-labeled detection molecule able to generate a colored deposit upon reaction with said enzyme,
- optionally a color chart for the interpretation of the colored deposit, and
- 20 - optionally an instruction leaflet.

Optionally said kit may also contain a diluent solution, and/or, a fixation solution, and/or, a solution comprising a capturing molecule, and/or, a solution comprising a detection molecule, and/or, a solution comprising a substrate for the enzyme linked to the capturing molecule, and/or, standard solution, and/or, control solution.

The present invention relates to a diagnostic kit or an assay device as described above for use in testing/controlling/detecting clinical, i.e. human or animal, agricultural, environmental or food samples.

30

All cited applications for human purposes may also be applied for veterinary purposes. According to present invention, a test sample which can be analyzed using a method according to present invention can be chosen from a group comprising cell fractions, serum, whole blood, urine, plasma, i.e. for human or animal diagnostic testing; soil, mud, minerals, water, air i.e. for environmental testing; any food materials i.e. for food testing; or any other medium/suspension/hard material which can be used for one of these purposes.

35

The present assay device may be used for controlling/testing/determining food samples. Presence of bacteria, viruses, mycotoxins, toxins, residues of pesticides, residues of antibiotics, residues of chemical substances may be determined. Examples of bacteria are *Salmonella* sp.,
5 *E.coli* sp., *Staphylococcus* sp., *Clostridium* sp., *Campylobacter* sp. *Listeria* sp., *Streptococcus* sp.

The present invention illustrates the detection of *Salmonella* by the FT method of present invention. However, the FT method of present invention can be applied to detect other DNA/RNA comprising biological agents, such as viruses, mycobacteria, all types of prokaryotic and
10 eukaryotic cells. The examples and figure legends serve to illustrate the invention and are by no way to be understood as limiting the present invention.

Examples of toxins are endo- or exotoxins, thermostables or thermolabile. Examples of mycotoxins are aflatoxins type A&B, ochratoxin. Examples of residues of pesticides are PCB,
15 dithiocarbamates, propamocarb, benzimidazoles, organochlorides. Examples of residues of antibiotics are residues from beta-lactams, tetracyclines, macrolides, quinolones, sulfonamides, aminoglycosides but also antibiotics that are used as animal growth promoters such as bacitracin, tylosin, spiramycin, virginiamycin and avoparcin. For environmental purposes similar antigens may be controlled/tested/determined.

20 The present diagnostic kit or an assay device may be applied for the diagnosis and/or monitoring of treatment of diseases. Examples are the detection of viral infections (virus family: adenoviridae, coronaviridae, papovaviridae, retroviridae, etc.) bacterial infections (*Yersinia*, *Aeromonas*, *Pasteurella*, *Vibrio*, *Helicobacter* (*H. pylori*) species, etc.) and the diagnosis of acute
25 phase proteins (rheumatoid factors, CRP, serum amyloid A, etc.). Major examples of viral infection are HIV (human immunodeficiency virus) causing AIDS and HCV (Hepatitis C virus) infections resulting in cirrhosis and liver failure.

For example, according to present invention the diagnostic kit or the assay device may be
30 applied for the diagnosis and/or monitoring of treatment of auto-immune diseases induced by organ specific or non organ specific auto-antigens. Table 1 summarises the main organ and non-organ specific antibodies. Tables 2 and 3 list organ and non-organ specific auto-antigens. Non-organ specific auto-antigens generally induce an auto-immune response whereby different organs are involved.

Said non-organ specific (multisystem) auto-immune disease may be chosen from the group of diseases comprising systemic lupus erythematosus (SLE) and other rheumatic diseases, scleroderma with or without Crest syndrome, drug-induced lupus erythematosus (LE), polymyositis with or without scleroderma, primary Sjögren Syndrome, rheumatoid arthritis and
 5 other connective tissue diseases (see table 2).

Organ specific antigens primary induce organ specific auto-immune diseases. In such case clinical manifestations originally affect only one organ, e.g liver in case of Primary Biliary Chirrosis but the onset of the disease also may affect other organs.

10 Said organ specific auto-immune disease may be chosen from the group of diseases comprising Addison's disease, auto-immune haemolytic anemia, chronic active hepatitis, coeliac disease, Goodpasture's syndrome, Grave's thyrotoxicosis, Hashimoto's thyroiditis, idiopathic thrombocytopenic purpura, Juvenile-onset diabetes, late onset diabetes, lens induced uveitis,
 15 some male infertility, multiple sclerosis, myasthenia gravis, pemphigoid, primary biliary cirrhosis, pernicious anemia, primary myxoedema, , sympathetic ophtalmia, ulcerative colitis, vasculitis and Wegener's granulomatosis (see table 3) (Lemoine 1992; Humbel; Abuaf et al.).

20 According to the present invention, the diagnostic kit or the assay device can be applied for the diagnosis and/or monitoring of treatment of infectious diseases included by viruses, bacteria, molds, mycobacteria or parasites. Examples of antigens which can be detected in case of infectious diseases are HIV, HbsAg, HbsAb, HbeAg, HbeAb, HbcIgM, Malaria, Chlamydia, StrepA, H.pylori, Lyme, Salmonella, E.coli, Syphilis, TB, Dengue and Chagas.

25 According to the present invention, the diagnostic kit or the assay device can be applied for the diagnosis and/or monitoring of treatment of allergic diseases or intolerance manifestations induced by numerous allergens from grasses, weeds, moulds, foods, trees, epidermals and dust. More than 2000 potential allergens have been identified. Examples of above mentioned allergens are: sweet vernal grass, thimothy grass, cultivated oat pollen; common ragweed, western
 30 ragweed, dandelion, *Penicillium notatum*, *Cladosporium herbarum*, *Candida albicans*, chicken egg white, cow milk, crab, egg yolk; maple, alder, birch, hazelnut, oak; cat and dog epithelium, horse and cow dander; greer or bencard dust.

35 According to present invention, the diagnostic kit or the assay device can be applied for use in the testing of cardiac markers. Said cardiac markers may be chosen from the group comprising

myoglobin, creatine kinase and troponin. Said inflammatory markers may be chosen from the group comprising C-reactive protein and interleukins.

According to present invention, the diagnostic kit or the assay device can be applied for use in testing of bacteria and/or toxins. With 'testing' is meant detecting the presence/absence, increase or decrease of certain antigens. Toxins may include mycotoxins. Examples of bacteria to be detected are *Salmonella sp.*, *E. coli sp.*, *Listeria sp.*, *Clostridium sp.*, *Staphylococcus sp.*, *Campylobacter sp.*, *Mycobacterium*, *Streptococcus*, *Shigella sp.*, *Bacillus sp.*

The present invention also relates to a diagnostic kit or an assay device, according to the invention, for use in testing of tumor antigens. Examples of antigens which can be detected in case of tumors are AFP, PSA, CEA, CA-15-3 and ferritin.

Examples of antigens which can be detected, using the device or kit of present invention, in case of drug abuse are methamphetamine, barbiturates, benzodiazepine, amphetamine, morphine, THC, cocaine and profile.

Generally, the present invention also implies a method using a diagnostic assay device according to the invention for the detection of an analyte present in a test sample.

Said method may comprise steps wherein the addition of the first analyte-binding compound, sample, second analyte-binding compound, substrate solution and fixation solution are performed subsequently, one after the other. Interestingly, no washing step is needed after the addition of the substrate. The omission of washing step is due to the specific blocking process of the membrane after the coating of the binding protein onto the membrane which block any remaining free site.

Alternatively, said method may comprise steps wherein the addition of the first analyte-binding compound, sample, second analyte-binding compound, substrate solution and fixation solution are not all performed subsequently, some of these may be premixed in advance before bringing them onto the device. The foregoing has stressed the application of the invention to sequential immunometric assays with monoclonal antibodies, i.e., an immunoassay using a first monoclonal antibody receptor on the porous member and a second monoclonal antibody receptor which is labeled. Sample is added to the porous member followed by labeled antibody. Other assay variants are possible. For example, in the case of an immunometric assay, the labeled antibody and sample may be admixed prior to addition to the porous member. In another embodiment of

the invention, the assay device is used to perform competitive assays, i.e., assays in which the first analyte-binding compound is bound to the porous member and for which the analyte in the sample competes with a fixed quantity of labeled analyte added to the sample solution or added following sample addition. Competitive immunoassays are conveniently run in this fashion using an antibody, for example, a monoclonal or polyclonal antibody preparation as first analyte-binding compound bound to the solid phase. Labeled antigen can be added to the sample before the sample is added to the porous layer. Alternatively, it can be added subsequent to addition of the sample or concurrently therewith. When a capturing molecule is used, the sample may be assayed in a variety of ways. For example, in a "sandwich assay", a first analyte-binding compound and a second labeled analyte-binding compound may be combined with the sample to bind the analyte prior to addition to the porous member. Alternatively, a first analyte-binding compound and a sample may be combined prior to addition to the porous member, or added in the sequence of first an analyte-binding compound and then sample, to be followed by addition of a labeled second analyte-binding compound. In such sandwich assays, the capturing molecule is selected to bind the first analyte binding compound and not the labeled second analyte-binding compound. In a competitive assay format, the intensity of the coloration of the deposit is inversely proportional to the concentration of the marker to be detected.

The assay device of the present invention also relates the use of a fixative solution obtainable as described in present invention. Such fixative solution allow to stop the enzymatic reaction and allow the permanent retain of results. As other rapid flow through tests are based on the detection of gold particles the use of a fixative solution is not necessary. This becomes only essential when an enzymatic-based step is introduced.

The present invention also describes a method for coating the intermediate porous layer of the device of the present invention comprising following steps:

- cutting membranes into strips
- immersing said strips into an application buffer and the capturing agent or first analyte-binding compound,
- incubating the membrane,
- immersing the membrane in a blocking agent containing 0.2 to 10% blocking agent, whereby the blocking agent may be BSA or any other agent known to block free sites on membranes,
- incubating membranes,
- drying strips, and,

- when storage is needed, the strips are packaged in order to protect membranes from humidity. This is preferentially realized by applying vacuum. Subsequent storage is performed,

wherein the coating (application) buffer features preferentially very low salinity and basic pH (9.1+/-0.1) the analyte-binding compound is present in an excess. The structure of the binding protein may require other types of application buffer.

According to a preferred embodiment of the present invention the intermediate porous layer of the device according to the invention is coated comprising following steps:

- 10 - cutting membranes into strips of preferably 0.8 cm wide,
- immersing said strips into a bath brought at RT containing the application buffer and the capturing molecule or the first analyte-binding compound,
- incubating the membrane for 3 hours at RT under gentle agitation,
- immersing the membrane in a blocking agent containing 1% BSA,
- 15 - incubating during 3 hours at RT under gentle agitation,
- drying strips at 37°C in an incubator for 1 hour to overnight, and,
- when storage is needed, packaging of the strips in order to protect membranes from humidity and stored at RT;

wherein the coating (application) buffer features preferentially very low salinity and basic pH (9.1+/-0.1), the analyte-binding compound is present in an excess. All parameters described here might be varied using conditions for the analyte to be detected by the flow-through assay device.

Preferentially the flow through method using a device as described by the invention is performed using the following steps:

- 25 - dilute sample 1/2 up to 1/100.000 into the diluent buffer, whereby the diluent buffer is a Tris buffer of low salinity and containing 1 to 5% BSA,. The composition of the diluent may be adapted to the analyte to be detected. Alternatively, an undiluted sample may be used.
- applying 15 µl or one drop) of diluted sample on the membrane,
- allowing the sample to soak for 1 minute at least, i.e. range from 30" up to 1'30" preferentially 30 45". The first soaking time depends upon the sample to be tested, for example a sample containing a high level of leukocytes or fibrin may soak more slowly,
- applying 25 µl or one drop of conjugate, i.e. anti-CRP coupled to HRP and allow to soak,
- applying 25 µl or one drop of precipitating TMB and allow to soak,
- applying 25 µl or one drop of fixative solution and allow to soak,
- 35 - waiting for 2 minutes before reading the result and read within 30 minutes, and,

- covering the colored spot with a scotch band, i.e. type 3M when a long-term storage of the result may be necessary.

As described in the example section, the present invention (example 1) also describes an optimized method for detecting analytes via dot blot based on an enzymatic assay as previously described for the flow-through concept. In this procedure coating a porous layer strip with a first analyte-binding compound or with a capturing molecule comprises the following steps:

- rinsing the membrane strips in alcohol for 1 to 60 seconds,
- incubating the membranes in a cold saline buffer between 1 to 60 minutes,
- 10 - adding 0.1 to 10 μ l coating solutions,
- incubating the strips for 1 hour to overnight between 4 to 25 °C under agitation,
- drying the membranes for 1 hour to overnight at room temperature
- saturating the membranes with a blocking protein during 15 min to overnight at RT or 4°C, respectively, under agitation, and,
- 15 - drying the membranes at 37°C overnight or over weekend.

These strips can be subsequently used in a dot blot or in any equivalent assay procedure.

The dot procedure can be performed comprising following steps:

1. diluting the analyte containing samples 1/2 or 1/10.000 into the Tris diluent buffer,
- 20 2. incubating 1 to 2 ml of this diluted sample for 5' to 45' (time X) at room temperature (RT) under agitation,
3. washing the membranes at least three times with 1 to 3 ml of Tris (0.01M) & Tween 20 (0.5% to 1%) solution for at least 3' each under agitation,
4. adding 1 ml to 2 ml of enzyme-labeled conjugate for which the dilution factor is adapted according to the method,
- 25 5. incubating the membrane for at least 5 to 30 minutes at RT (time Y) under agitation,
6. washing the membranes three times for at least 3' each under agitation,
7. adding 1 to 2 ml of chromogen solution (membrane precipitating substrate),
8. incubating the membrane for 1' to 15' (time Z) at RT under agitation,
- 30 9. stopping the reaction by addition of 1 ml of stopping solution,
10. analysing the appearance of a blue spot of variable intensity.

All parameters described here might be varied using conditions as described for the above developed flow-through assay device.

- 35 The following examples and figure legends merely serve to illustrate the invention and are by no way to be understood as limiting the present invention.

Brief description of the figures

Table 1: Main organ and non-organ specific antibodies present in autoimmune diseases.

5 Table 2: non organ-specific antigens.

Table 3: organ-specific antigens.

10 Table 4 (4a-4c): Evaluation of the flow-through method as described in the present invention and other existing methods of immunoturbidimetry and flow-trough method from Nycomed.

Table 5: Evaluation of different membrane lots.

15 Table 6: Testing of different lots of membranes.

Table 7 (7a-7h): Evaluation of intra-assay reproducibility.

Table 8 (8a-8f): Evaluation of the inter-assays reproducibility.

20 Table 9 (9a-9c): Evaluation of different TMB agents.

Table 10 (10a-10c): Evaluation of the lowest detection which can be detected.

25 Table 11 (11a-11b) : Detection of anti-mitochondrial type M2 antibodies.

Table 12: Detection of Salmonella thyphimurium.

Table 13: Influence of the composition of the diluent buffer.

30 Table 14 (14a-14e): Correlation with another rapid method for the detection of CRP by immunoturbidimetry.

Modes for carrying out the invention

35 The inventors developed a membrane based rapid test for the detection of analyte(s). The described test has been first developed and optimized for the detection of the *C-reactive protein*

(CRP) which is an acute phase protein (examples 1-4, 8, 9, 16). As the inventors proved in the present invention, this test can be used to detect any other analyte in any other media.

Other assays also have been performed for the detection of rheumatoid factors (example 6), M2
5 antibodies (example 5) or *Salmonella typhimurium* (example 7).

In experiment 1 the presence of the analyte CRP was studied using a polyclonal antiserum anti-CRP (from rabbit origin) at a final concentration of 10µg/ml as capture antibody during the coating procedure.
10

In example 5 the presence of anti-M2 antibodies was studied; a purified organ specific auto-antigen pyruvate dehydrogenase (PDH antigen =M2 antigen) with an enzymatic activity of 0.0634 units/ml was coated.

15 In example 6 the presence of rheumatoid factors was analysed using purified human immunoglobulins as capture antibody during the coating procedure; a solution of purified human immunoglobulins with a coating concentration of 48 µg/ml was applied.

In example 7 the presence of *Salmonella typhimurium* was studied using a monoclonal antibody
20 as capture protein during the coating procedure at a final concentration of 0,1 mg/ml.

The serum or plasma levels of CRP rise in response to infectious and non-infectious inflammatory processes. The levels may rise from +/- 5 mg/L to 500 mg/L. Measurements of the CRP level are useful in distinguishing viral, i.e. mild increase up to 50 mg/L from severe bacterial
25 infections, i.e. up to 500 mg/L and to check the efficiency of treatment. Detection of low levels of CRP ranging from 1 mg/L to 5 mg/L may be predictive for atherosclerosis.

Several methods can be used for the detection of CRP, such as Radio Immunoassay (RIA), Radial immunodiffusion, Latex agglutination, Turbidimetry, Nephelometry, Enzyme Immuno
30 Assay, e.a. Enzyme Linked Immunosorbent Assay, Fluorescence polarimetry and Membrane based immuno-assays. Radial immunodiffusion or latex agglutination methods only allow a yes or no or semi-quantitative interpretation. These methods in general suffer for a lack of sensitivity or lack of precision. Other methods mentioned above are instruments based tests allowing a quantitative interpretation of results. However for such methods the time required to get the
35 results is generally too long and often the procedure require several washing steps.

Therefore new reliable rapid test systems are needed for the diagnosis of especially inflammatory diseases.

Example 1: Optimization of a dot method as rapid test.

- 5 The aim of this feasibility study was to develop a rapid method allowing the interpretation of the results within a few minutes without any sophisticated lab instrumentation.

1.1 Manufacturing of dot strips

- 10 PolyVinylideneFluoride membranes from Millipore (cat. 00010 IPVH) with a pore diameter of 0.45 μm were used and cut into strips of 3 mm wide. Strips were rinsed with absolute ethanol (Merck 1.00.983) for 15 seconds and incubated in cold TBS (Tris buffer saline, 10mMTris, 150mM NaCl, pH 9.0+/-0.1) during 10 minutes before starting the coating (application) procedure. The coating volumes are small, i.e. 1 to 5 μl and requires an accurate pipettes or accurate spotting device, e.g. BIO dot system. In this experiment an accurate pipette was used for this purpose. After the coating using a "spotting procedure", the membranes were dried at 37°C followed by a saturation step using a blocking protein ranging from 1% to 5% in concentration. In this experiment BSA was used. This blocking step was performed for 1 hour at RT under agitation. The membranes were subsequently dried overnight at 37°C.

- 20 Three experiments were performed using these coated strips whereby for each experiment a different analyte was searched for and/or a different antibody was used.

1.2 Dot procedure

According the analyte to be detected, the dot procedures were performed as follows:

- 25 1. The samples containing CRP protein –experiment 1- or rheumatoid factors type IgM –experiment 2 - in anti-M2 antibodies –experiment 3 - were diluted 1/50 to 1/100 into Tris diluent buffer
2. 1 to 2 ml of this diluted sample was incubated for 5' to 45' (time X) at room temperature (RT) under agitation.
- 30 3. The membranes were washed three times using 1 to 3 ml of Tris (0.01M) & Tween 20 (0.5% to 1%) solution for 3' each, under agitation.
4. 1 ml to 2 ml of conjugate was used for further incubation.
5. Incubation was performed for 5 to 30 minutes at RT (time Y) under agitation.
6. The membranes were washed for a second time three times for 3' each under agitation (see step 3).
- 35 7. 1 to 2 ml of chromogen solution, i.e. membrane precipitating TMB was added.

8. Incubation was performed for 1' to 15' (time Z) at RT under agitation.

9. The reaction was stopped by the addition of 1 ml of stopping or fixative solution
10. The appearance of a blue spot of variable intensity was visually analysed.

5 The conjugate's specificity was chosen according to the analyte to be detected. In experiment 1 the HRP labeled anti-CRP was used at concentration of 0.65g/L; in experiment 2 the human anti- μ chain IgM (P0322 from Dako, Fab'2) was used at concentration of 1 g/L; in experiment 3: a mix of human anti-gamma chain IgG (P406 from Dako, Fab'2) and human anti- μ chain IgM (P 322 from Dako, Fab'2) was used at a final concentration of 1 g/L.

10

For all three experiments the composition of the fixative solution was as follows (for 100 ml):

0.1 g dioctyl-sulfosuccinate	SIGMA	D 0887
0. 5 ml dimethylformamide	"	D 8654
1.9 ml polyvinyl alcohol 5%	"	P8136

15

76 ml H₂O (filtrated)

1.3 Interpretation of results

The color formation was qualitatively evaluated. When a color was formed it was interpreted that the sample is positive, meaning that the test sample contains the analyte.

20

1.4 Final conclusion

Precipitating TMB allowed the development of a blue coloration proportional to the concentration of CRP. Presence of solubilization reagents in the diluent buffer seemed to avoid interference due to other blood protein such as human albumin and immunoglobulins. The coating procedure and the blocking procedure were optimized decreasing the background. The use of new dot
25 strips allowed good results with 5'5'1' incubation times using the enzymatic detection HRP-TMB system. Nevertheless, washing steps were still cumbersome and simple dippings which excludes the use of any washing steps and decreased incubation times 2'2'1' induced too high background.

30

In addition, with such method, a gentle agitation is required in order to optimize the immunologic interaction (Ag-Ab). Also, washing steps are obligatory in order to separate the free fraction from the bound fraction. Moreover, this washing procedure may be performed under agitation in order to avoid background such as blue background coloration of the strips.

35

Finally, this method requires some equipment lab, e.g. rocking agitator and some procedure steps are cumbersome, e.g. washing procedure. Moreover, large volumes of reagents may be used, i.e. 1 ml or more which do not meet the volume requirements for the rapid tests (see introduction).

5

Due to the above-mentioned arguments, the requirements related to the dot procedure did not meet the specifications of rapid tests.

Technical parameters which should be improved:

- 10 1. incubation times
 - the target time is 5 min total eg 2':2':1'
 - a titration of the conjugate may be performed in order to avoid false positive or false negative results
 - current sensitivity (5':5':1') is +/- 10mg/Liter but should be improved
- 15 - blue coloration allows a good visual discrimination, a semi-quantitative determination is possible.
2. washing steps: too cumbersome.
3. test design in order to avoid lab equipment

20 **Example 2: optimization of the flow-through concept**

During the development of a rapid test for the detection of CRP, the inventors focused on the optimization of following technical parameters: composition, quality and pore size of the membrane (see 2.1), optimization of the enzyme labeled reagent system to detect the analyte (here CRP) (see 2.2), composition of the coating buffer (application buffer) (see 2.3), composition and titration of the analyte-binding compound agent, here anti-CRP HRP labeled antibody, application of the binding procedure (coating procedure), blocking procedure (post-coating procedure) (see 2.4), storage/stability of the membrane, stability of the result retaining of (coloration) and costs.

30 **2.1 Selection of the membrane (composition – pore size)**

Quality and composition of membrane do exert an influence on the binding of the capturing agent or the first analyte-binding compound (anti-CRP antibody) and the flow rate (sample, conjugate and precipitating substrate). It is thus important to achieve a high consistent binding of the protein in order to get a very good sensitivity, a high specificity and a good reproducibility. Also the pore diameter of the membrane is of great importance.

35

The inventors found that membranes with pore-diameters of 1.2 μm and 0.8 μm induce a too fast flow rate (within a few seconds) which give a diffuse colored spot difficult to interpret.

Brief description of the experiments

5 Membranes from S&S with a pore diameter of 1.2 μm and membrane from mdi with a pore diameter of 0.8 μm were coated via a spotting procedure using 3 or 5 μl of an anti-CRP antibody. A sample positive in CRP (concentration 40 mg/L) was diluted 1/25 into a Tris diluent buffer (10mM) containing 1% sucrose. The inventors noticed that a too rapid, i.e. less than 10 seconds flow rate and a large diffusion process. Moreover, after addition of the conjugate, i.e. anti-CRP antibody HRP labeled) the background, i.e. blue background coloration was too intensive in order to allow a good visual reading. The background coloration was tested by the use of the diluent buffer only. In contrast, a membrane from mdi with a pore diameter of 0.45 μm showed acceptable background signals and was subsequently selected. Here the flow rate was slower, i.e. +/- 30 seconds.

15 In addition, a smaller pore size, i.e. 0.45 versus 0.8 and 1.8 μm induces a greater surface area, thus a higher binding capacity. Also, a small pore size allows to avoid the radial diffusion of the reagents during the flow through resulting in the formation of a well shaped colored spot.

20 The final selection was made between type CN and type CLN provided by the company mdi (Advanced Microdevices (PVT) LTD. 21, Industrial Area, Ambala Cantt, 133 001 India), both nitrate cellulose membranes.

25 Membrane type CN, is a plain membrane which is not supported and is relatively fragile. The inventors observed heterogeneous colorations probably due to air pockets that developed between the absorbent pads and the membrane. Moreover, the flow rate for sample was "too slow", more than 3 minutes.

30 Membranes type CLN are supported, paper backed meaning "supported" by paper, easy to handle and induce the appearance of a homogeneous coloration. Because the nature of support, there is always a good contact between the absorbent and the membrane, i.e. no air entrapment. Further, the structure helps to regulate the flow rate of the membrane to a low value resulting in an enhanced sensitivity. The CLN-040-SL53 has a pore size of 45 μm , a thickness of approximately 480 μm and a protein binding capacity of 103 $\mu\text{g}/\text{cm}^2$.

35

The absorbent pad (AP 120) used in the absorbent layer has a special property and is partial swelling on wetting in order to ensure good contact with membrane. This pad was bought from the same company mdi and has a thickness of approximately 1550 µm.

5 *2.2. Selection of the labeled system*

The method was optimized such that an easy visual interpretation of results, meaning a clear colored signal for the positive samples but also a very low signal for the negative samples, i.e. low background. Also, the visual discrimination between low-positive results observed in case of viral and mild-bacterial infections, i.e. CRP concentration up to 50 mg/L and strong positive results observed during severe bacterial infections, i.e. from +/- 60 up to 200 mg/L had to be clear. A reproducible test system, i.e. consistent results within different batches of membranes was obtained. Indeed, the determination of CRP concentration is useful in order to evaluate the follow-up of the treatment, i.e. antibiotics. An assay device is of low cost.

15 The end formed visual signal on membrane based tests can be formed by colored latex particles, gold particles, dyes or enzymes. The use of latex particles may lead to problems of interpretation and does not allow a permanent result. Self-agglutination also may occur, affecting the specificity of the method. Gold particles of good quality are relatively expensive. Some problems of reproducibility manufacturing in terms of spherical shape, size also may be encountered.

20 Literature showed that the size of gold particles was a critical factor.

The present inventors decided to use a conjugate, i.e. purified anti-human CRP antibodies from rabbit origin coupled to Horse Radish Peroxidase –HRP- and a TMB precipitating system. Contrary to the use of gold particles, the presence of CRP is thus revealed by a one-step enzymatic reaction which is stopped by the use of a fixative, i.e. stopping solution.

The use of a fixative solution allows a permanent record of the result. This is not the case with gold particles for which the reading may be performed within 5 minutes and for which the result cannot be retained. The enzymatic reaction consists of the reaction of 3-3',5,5'

30 tetramethylbenzidine with horse radish peroxidase and hydrogen peroxide forming a blue free radical cation 1-electron oxidation product.

Advantages of such combination include commercial ready to use TMB preparations of high consistent quality and with a long shelf life are available on the market. Manufacture of such reagent is easy and reproducible. The use of such precipitating system allows the development of a blue, i.e. aquamarine coloration easy to read. The conjugate used in this experimental setup

35

can be titrated so that the best dilution of this compound can be selected allowing a correct, reproducible and accurate reading. In combination with a reflectance reader this assay device allows an accurate standardization of each batch of membrane, which guarantees a good reproducibility.

5

2.3 Optimization of components

Membrane

The membrane is the most critical factor in order to get a good test result. Even if exact mechanisms of binding are still unknown, e.g. hydrophobic interactions, hydrogen bonding, electrostatic interactions, high binding level is obtained through optimized application procedure and application (coating) buffer. Nitrocellulose is preferably chosen due to its neutral charge.

10

In the coating procedure membranes were cut into strips of 0.8 cm wide as it fitted to the flow-through device and slowly immersed into a bath brought at RT and containing the application buffer and the analyte-binding compound. It can be remarked that if the strips are immersed into a cold application buffer, e.g. 4°C strips tend to "retract" and the binding efficiency is decreased.

15

The coating (application) buffer features very low salinity and basic pH (9.1 \pm 0.1) character. The first-analyte binding compound was applied in excess using a polyclonal antiserum anti-CRP with high affinity. An antibody stock having a titer concentration of 10.0 mg/ml \pm 1.0 was diluted 1/50 in the application solution resulting in a binding concentration of 0.2 mg/ml.

20

Composition of application buffer for 1 liter

1.2 g Tris	ICN 103133
8.8 g NaCl	Merck Eurolab (ME)1723

25

Several experiments showed that the reactive zone (cellulose nitrate) may be in direct contact with the application solution. Immersion gave the best results. A spotting coating procedure method gave less good results due to the formation of inhomogeneous and diffuse spots.

30

Several incubation, i.e. immersion times have been tested from 1 hour to 5 hours. An incubation of 3 hours at RT (18-22°C) under gentle agitation (rocking agitator) gave the best results.

After the application, membranes are not washed off in order to avoid "desorption" of the analyte-binding compound due to the presence of surfactant agents in the washing buffer. Indeed, presence of a detergent such as Tween 20 which is usually present in the wash buffer might cause this effect.

35

2.4 Post-coating – blocking -procedure:

Such procedure was introduced in the concept and optimized in order to get a low background but also in order to avoid a washing step usually necessary during the FT (flow through) method.

- 5 The aim was to block any free remaining sites on the membrane. The post-coating or blocking procedure is widely used in the ELISA methods, i.e. using soluble substrates in order to block the remaining free sites after the coating procedure.

- 10 The strips used in the dot method are generally, but not always, blocked in order to avoid a too high background induced by the non-specific adsorption of the conjugate onto the strip. In FT methods washings are obligatory in order to eliminate the unbound gold particles conjugate, i.e. gold antibody conjugate which may be adsorbed onto the membranes.

- 15 In the method according to present invention, the remaining free sites after the coating procedure are blocked using very purified BSA (bovine serum albumin) at a concentration of 1% in order to prevent any non-specific adsorption of the conjugate during the flow-through. Nevertheless, other proteins or polymer components such polyvinyl alcohol, polyvinyl pyrrolidone may be used for the same purpose.

- 20 After the coating procedure, strips are thus immediately immersed into a second bath containing the blocking agent. Strips are placed under gentle agitation (rocking agitator) during 3 hours at RT (18-24°C).

Composition of the blocking buffer (for 1liter)

- | | | | |
|----|---|---------------|--------|
| 25 | 8.5 g NaCl | MERCK EUROLAB | 1723 |
| | 1.25 g Na ₂ HPO ₄ | " | 1770 |
| | 0.160 g NaH ₂ PO ₄ .2H ₂ O | " | 1769 |
| | 1g thimerosal | " | 818957 |
| | 10 g BSA | ICN | 105133 |

30

Due to the high binding of the analyte-binding compound onto the membrane this compound was not "displaced" by the BSA which blocks the remaining free sites. Strips were immersed into the blocking solution during 3 hours under gentle agitation. Thimerosal was added as a preservative.

- 35 Also after the blocking procedure, membranes were not washed off in order to avoid "desorption" of the analyte-binding compound and the blocking agents due to the presence of surfactant

agents. Indeed, presence of a detergent such as Tween 20 which is usually present in the wash buffer might cause this effect.

5 Strips were dried at 37°C during 1 hour and then placed overnight in an incubator at 18-22°C in order to avoid dust. The drying step may also be performed overnight at 37° without having any affect on the final result. Strips were then packaged in order to protect them against humidity under vacuum and stored at RT.

10 The handling procedure described above gave the best results with the flow through procedure described hereafter (see section 2.5).

The analyte-binding compound is used in excess; the application buffer supplemented with the analyte-binding compound may be reused 2 times if frozen at -20°C between two handling steps.

15 Similarly, the blocking solution also may be reused 2 times within 1 month, if stored at 4°C, between 2 handling steps without affecting the efficiency of the blocking procedure.

2.5. FT procedure

20 The sample, i.e. serum or whole blood was diluted 1/50 into the diluent buffer. The diluent buffer used was a Tris buffer of low salinity and containing BSA, i.e. 3 or 5%. The dilution was performed whereby 10 µl of the blood sample was added to the test tube containing 500 µl of diluent buffer. This test tube as subsequently closed and mixed.

Preparation for 1 liter diluent buffer

25	1.2 g Tris	ICN	103133
	8.8 g NaCl	ME	1723
	50 g BSA ref.	ICN	105033
	1 g thimerosal	ME	818957

30 15 µl or a drop of diluted sample was applied onto the membrane and the sample was allowed to soak through the membrane for at least 1 minute. Usually, the absorption time is around 45", i.e. range 30" up to 1'30". Such time is slower than those observed with other FT method. It is known that the sensitivity of the test increase with decreasing flow rate. Indeed, too fast flow rates, i.e. a few seconds does affect the sensitivity of the method.

This was followed by the addition of 25 µl or one drop of conjugate, i.e. anti-CRP coupled to HRP, 25 µl or one drop of precipitating TMB, 25 µl or one drop of fixative solution. After each addition, the solution was allowed to soak through the membrane. The result was read after minimal 2 minutes. Indeed, it may be necessary to wait at least 2 minutes before stopping the enzymatic reaction. For a long-term storage of the result, it is advised to cover the coloured spot with a scotch band, i.e. type 3M.

The procedure with the mentioned volumes 15-25-25-25 µl induce a homogenous colored spot of 3-4 mm of diameter. If a bigger diameter must be obtained > 4 mm or if volumes are higher, e.g. droplet dispenser of 50µl, the composition of diluent buffer has to be modified by the addition of sucrose, i.e. 10% up to 40% which slow the flow rate and let avoid heterogeneous coloration.

Composition of the fixative solution (for 100 ml)

1 g dioctyl-sulfosuccinate	SIGMA	D 0887
5 ml dimethylformamide	"	D 8654
19 ml polyvinyl alcohol 5%	"	P8136
76 ml H ₂ O (filtrated)		

2.6. Interpretation of result

2.6.1 visual reading = semi-quantitative reading

The CRP concentration was estimated by comparing the test response with a standard curve prepared with highly purified CRP. The six zones of the chart correspond to the following concentrations: 11 mg/L, 27.5mg/L, 69mg/L, 91mg/L, 137 mg/L and 275mg/L. Alternatively, other concentrations covering the range from 1 mg/L to 500 mg/L may be used.

Samples were interpreted as follows:

Color of test response	CRP concentration
- lighter than the 11mg/L zone	< 11 mg/L
- identical to a zone	in agreement with the zone
- between 2 zones	estimate a value between the zones
- darker than the 275 mg/L zone	> 275 mg/L

In the kit, a colour chart will be included allowing the comparison of the coloration of the sample with the coloration of known concentrations of CRP. Alternatively, the colored spots might be scanned using a reflectance reader as discussed below.

2.6.2 Interpretation with use of a reader

Using a reflectance reader measurement of the intensity of the coloration is more accurate and allows to determine the concentration in CRP. The concentration of an unknown sample can be calculated by interpolation on a standard curve obtained with several concentrations of purified CRP.

Example 3: Comparison with other existing method.

3.1. Aim of the study

The optimized FT method was compared with 2 commercially available methods.

Experimental conditions

The inventors analysed 50 serum samples ranging from very low to high concentration in CRP which have been tested by the nephelometry method (quantitative method) using the Bio ART's flow through method, i.e. semi-quantitative method applying precipitating TMB and using the Nycomed method, i.e. semi-quantitative FT method applying gold particles.

3.2. Procedure

Samples have been tested according to the following procedure:

a) FT BIO-ART (according to present invention):

1. Using a pipette, 15µl of diluted sample (1/50) was applied onto the coated membrane and allowed to soak for at least 45 seconds.
2. Using a pipet (see table 4a) or a droplet dispenser (see table 4b) 25 µl of conjugate, i.e. anti-human CRP from a rabbit origin HRP labeled with horse radish peroxidase (HRP) with a concentration of 0.9 mg/L was applied to the coated membrane and allowed to soak.
3. Using a pipette (see table 4a) or a droplet dispenser (see table 4b) 25 µl of chromogen (precipitating TMB) was applied to the coated membrane and allowed to soak.
4. Using a pipette (see table 4a) or a droplet dispenser (see table 4b), 25 µl of fixative solution was applied to the coated membrane and allowed to soak.
5. Visual reading and/or measurement of the reflectance was performed after 2 minutes of stabilization.

b) FT Nycomed:

The kit from Nycomed has been used according the recommended procedure described in the package insert. Kit with lot n°10092464 was used.

1. Using a pipette, 25µl of diluted sample sample (1/40) or positive control was applied to the coated membrane. The sample was allowed to soak for at least 45 seconds.

2. One drop of conjugate was applied to the coated membrane and allowed to soak.
3. One drop of washing solution was applied to the coated membrane and allowed to soak
4. Reading, i.e. visual or by the use of the reflectance reader was performed after 30 seconds of stabilisation.

5

In order to validate the reflectance reader, both BIO ART and Nycomed results were read with the reflectance reader. Using a standard curve, the concentration of the sample was calculated in order to foresee the feasibility of a real quantitative method.

10 **3.3. Reagents:**

membrane lot n° : 00 6 4 (table 4a) and 00 1 15 (table 4b)

precipitating TMB lot n° : 5 DT 0629 Exp. Date : 06/01

fixative solution lot n°: 0051 Exp. Date : 05/01

conjugate : anti-CRP –HRP labeled diluted 1/700 (0.9mg/L)

15

3.4. Interpretation of results
3.4.1 Nycomed : visual reading = semi-quantitative reading

The CRP concentration was estimated by comparing the test response with the reference color chart provided with the kit. The five zones of the chart corresponded to the following concentrations: 10 mg/L, 25mg/L, 50mg/L, 100mg/L and 200mg/L.

20

Signals were interpreted as follows:

Color of test response	CRP concentration
- lighter than the 10mg/L zone	< 10 mg/L
- identical to a zone	in agreement with the zone
- between 2 zones	estimate a value between the zones
- darker than the 200 mg/L zone	> 200 mg/L

25

3.4.2. BIO ART: visual reading = semi-quantitative reading

The CRP concentration present in the samples was estimated by comparing the test response with a standard curve prepared with highly purified CRP. The six zones of the chart representing the standard curve corresponded to the following concentrations: 11 mg/L, 27.5mg/L, 69mg/L, 91mg/L, 137 mg/L, 275mg/L. Other concentrations covering the range from 10 mg/L to 250 mg/L may be used.

35

Interpretation was guided using following criteria:

	Color of test response	CRP concentration
	- lighter than the 11 mg/L zone	< 11 mg/L
	- identical to a zone	in agreement with the zone
	- between 2 zones	estimate a value between the zones
5	- darker than the 275 mg/L zone	> 275 mg/L

3.4.3 Calculation of results with the reflectance reader

The end-coloration was read by using the reflectance reader as described hereafter.

The CRP concentration was calculated from the standard curve. Results are expressed in mg/L as listed in tables 4a and 4b.

3.5. Results :

See tables 4a and 4b.

3.6. Conclusions :

From table 4a it may be concluded that there is no discrepancy in terms of negative versus positive result, 3 samples * (30, 34, 43) are classified into different classes and according to the lot membrane, 5 samples** (25/26/30/35/37) are differently classified. In order to always get the same signal for the same level of concentration, the conjugate may be titrated, e.g. this titration gives allows to determine the best final dilution that might be used in the respective experiment.

In table 4b the first results show that the quantification is feasible. As the concentration is calculated by interpolation of the standard curve, no accurate concentration can be calculated for values lower than 10 mg/L. Additional standards, i.e. with values of 2 and 5 mg/L are needed in order to calculate the concentration with accuracy. For samples varying between 10 to 50 mg/L, correlation between both methods is good. Sample n° 31 has to be tested again For samples varying between 51 to 150 mg/L, correlation is also good except that sample 39 has to be tested again. For the visual reading (see table 1a) the inventors noticed that both the Nycomed and method according to present invention lacks discrimination for samples having an analyte concentration higher than 150 mg/L. By adjustment of the conjugate's dilution, such problem can be overcome. By adjustment of the TMB concentration, such problem can be overcome.

Further to this preliminary study, reagents have been optimized and a second series of samples with concentrations ranging from 0,4 mg/L to 200 mg/L have been tested according the BIO ART FT procedure. Results were compared with Nycomed method and with nephelometry. A standard curve prepared with purified CRP was prepared in order to interpret the results. The six

concentrations of the Bio ART standard curve were 0 mg/L, 1 mg/L, 10 mg/L, 25 mg/L, 75 mg/L, 200 mg/L.

a) FT BIO-ART (according to present invention):

- 5 1. Using a pipette, 20µl of diluted sample (1/50) was applied onto the coated membrane and allowed to soak for at least 45 seconds.
2. Using a droplet dispenser (see table 4c) 25 µl of conjugate, i.e. anti-human CRP from a rabbit origin HRP labeled with horse radish peroxidase (HRP) with a concentration of 0.9 mg/L was applied to the coated membrane and allowed to soak.
- 10 3. Using droplet dispenser (see table 4c) 25 µl of chromogen (precipitating TMB) was applied to the coated membrane and allowed to soak.
4. Using a droplet dispenser (see table 4c), 25 µl of fixative solution was applied to the coated membrane and allowed to soak.
5. Measurement of the reflectance was performed after 2 minutes of stabilization.

15

The results are shown in table 4 c.

- The addition of a standard 1 mg/L allows a better discrimination in low values, i.e. < 10 mg/L. For samples varying between 10 to 75 mg/L, correlation between both methods is good. Sample n°
- 20 31 was tested again and showed a conc. of 43,8 mg/L which is more consistent with the result from nephelometry. For samples varying between 75 to 150 mg/L, correlation is also good except sample 39 which still showed a result lower than nephelometry (67 mg/L). As for the Nycomed method, value higher than 200 mg/L cannot be discriminated easily.

25 **Example 4 : evaluation of the reproducibility of results by the use of a reflectance reader**

4.1 Introduction

- The reader is an imaging reader allowing the quantification of the colored signal by reflectance. The reader has been adapted to be used to read the signals obtained by the flow through CRP method as described in present invention. This reader allows the measurement of a specific
- 30 area. This area is not limited to a specific form such as a circle.

- Advantages of such quantification are evaluation of the inter-batches reproducibility, titration of the conjugate in order to get the same signal for the same concentration, evaluation of the sensitivity, evaluation of the intra-assay reproducibility and standardization of the quality control
- 35 procedure (quantitative "acceptance/reject" specifications).

4.2.. Experiments conditions

The FT procedure has been performed according procedure described in example 3 and 4. For each experiment the membrane and sample used are indicated. The dilution performed for the conjugate is additionally mentioned.

5

a) Membrane lot n° 0064 sample 48 conjugate 1/700

The fourth lot of membrane produced in June (lot 00 6 4) was tested using a conjugate with a concentration of 0.9mg/L. A serum sample n° 48 having a concentration of 258 mg/L CRP was diluted 1/50 into the Tris diluent buffer containing 5% BSA and tested 5 times on 5 separate pieces of membrane (Replicate 1 (R1) to Replicate 5 (R5)). The procedure previously described (experiment 3 and 4) was used and reading was performed 2 minutes after fixation. The reflectance was measured using a red (R), a green (G) and a blue (B) filters. Results were interpreted according the measurement using the red filter since the difference in intensities between the different spots between 0 and higher values is more pronounced using this filter. The 5 replicates gave the following results: R1: 60.65 reflectance units (RU); R2 59.47 RU; R3 61.30 RU; R4 59.68 RU and R5 63.24 RU. The mean of the signals was 60.8 RU showing a standard deviation of 1.5 RU. The coefficient of variation (CV) was 2.5%.

20 b) Membrane lot 0093

The third lot produced in June (00 6 3) was tested under conditions as previously described for lot n° 0064. Results for the 5 replicates by using the red filter were: R1 66.72 RU; R2 70.05 RU; R3 68.10 RU; R4 68.54 RU and R5 68.02 RU. The mean of the signals was 68.2 RU with a standard deviation of 1.2 RU. The coefficient of variation was 1.8%.

25

c) Membrane lot 0094 sample 48 conj. 1/700

The fourth lot produced in September (00 9 4) was tested under conditions described for lot n° 0064. Results for the 5 replicates by using the red filter were R1 73.67 RU; R2 71.14 RU; R3 73.74 RU; R4 78.42 RU and R5 74.35 RU. The mean of the signals was 74.3 RU with a standard deviation of 2.6 RU. The coefficient of variation was 3.5%.

30

d) Membrane 0064 sample 30 conj. 1/700

The fourth lot produced in June (00 6 4) was tested under conditions described for lot n° 0064 but with an hemolysed sample (n°30) with a concentration of 52.7mg/L. A hemolysed sample contains remains of blood cells which might influence reading of the obtained signals. In this way, it was tested if contaminating compounds influence the reading of the assay. Results for the 3 replicates by using the red filter were: R1 76.38 RU; R2 67.60 RU and R3 78.56 RU. The mean of the signals was 74.2 RU with a standard deviation of 5.9 RU. The coefficient of variation (CV) was 7.8%. The inventors concluded that a higher CV as obtained with hemolysed sample using this method.

10

e) Membrane 0093 sample n° 30 (hemolysed) conj. 1/700

The third lot produced in September (00 9 3) was tested under conditions described for lot n° 0064 but with an hemolysed sample (n°30) with a concentration of 52.7 mg/L. Results for the 3 replicates by using the red filter were: R1 83.30 RU; R2 82.34 RU and R3 81.73 RU. The mean of the signals was 82.4 RU with a standard deviation of 0.8 RU. The coefficient of variation was 0.9%. From these results the inventors concluded that not the method "as such" but the membrane used for this method influenced the variation.

15

f.) Membrane 0064 sample 6 conj. 1/700

The fourth lot produced in June (00 6 4) has been tested under conditions described for lot n° 0064 but using sample (n°6) with a CRP concentration of 2.0 mg/L. Results for the 4 replicates by using the red filter were: R1 129.6 RU; R2 126.6 RU; R3 131.8 RU and R4 126.6 RU. The mean of the signals was 128.7 RU with a standard deviation of 2.5 RU. The coefficient of variation for this experiment was 1.96%.

25

g) Membrane 0081 sample 6 conj. 1/700

The first lot produced in August (00 6 1) was tested under conditions described for lot n° 0064 with sample (n°6) carrying a CRP concentration of 2.0 mg/L. In this experiment different volumes of reagents were tested and its influence of the detected signals analyzed.

30

g.1 Reagents volumes used: 15-25-25-25 µl

Signals that were obtained were: R1 122.7 RU and R2 137.1 RU.

g.2 Reagents volumes used: 15-15-15-15 µl

Signals that were obtained were: R3 141.5 RU and R4 150.6 RU.

The inventors observed a small difference in the Intensity of the coloration confirmed by the measurement of the reflectance.

h) testing of standard curve on different lot of membranes

5 *Experimental conditions*

A standard curve containing highly purified CRP was prepared in the Tris diluent buffer containing 3% BSA. The flow through procedure was performed according previous description (experiment 3 and 4) and the measurement of reflectance with the red filter was performed on several lots of membranes

10

Aim of the experiment:

The inventors observed a poor visual discrimination for membrane lots 00102 and 00107 and a good discrimination for lot 00103 and 00106. The coating procedure was different according lot
15 n° lot number and manufacturing (coating process). Lots 00103 and 00106 were made on strips of 0.8 cm wide; Lots 00102 and 00107 were made on larger strips 8.5 cm wide. According the quality control procedure, the accurate measurement of the reflectance will allow to accept or reject the membranes.

20 Results are shown in table 5. Sizes of membrane strips used are indicated. The reflectance units are shown for each standard concentration used.

Due to the lack of discrimination in the color intensity, membranes lot n° 00102 and 00107 were
25 rejected. Membranes 001030 and 00106 featured a good visual discrimination, confirmed by the measurement of reflectance and passed the quality control procedure.

i) testing of samples on different lots of membranes

Experimental conditions

30 Samples with various concentrations in CRP were tested according the FT procedure as previously described (experiment 3 and 4) in order to confirm which size of membrane strips are optimal to use to get the best color discrimination.

Following samples were used and diluted 1/50 into Tris diluent buffer with 3% BSA: sample n° 45
35 (208 mg/L); sample n° 41 (157 mg/L); sample n° 37 (94 mg/L); sample n° 29 (48 mg/L); sample n° 21 (20 mg/L) and sample n° 9 (15 mg/L). .

Results are presented in table 6. Sizes of the strips used are indicated; reflectance units for each concentration tested are mentioned for each tested membrane.

- 5 The inventors concluded that there exists no relationship between the size of the strips during the coating process and the quality of results.

j) Evaluation of the intra-assay reproducibility additional data

Preliminary experimental conditions

- 10 The FT was performed according the previous description (experiment 3 and 4). 3 samples carrying different concentrations in CRP have been tested in 4 replica. The reflectance was measured and the coefficient of variation calculated.

Following samples were used and diluted 1/50 into Tris diluent buffer supplemented with 3% BSA: sample n° 41 (157 mg/l); sample n° 29 (47.8 mg/L) and sample n° 15 (9 mg/L).

15

Results are presented in table 7a. Reflectance units are indicated for each sample used. The mean of the signals determined, the standard deviation and the coefficient of variation for each group of measurements are shown.

- 20 *Experimental conditions additional data*

Further to this, extensive tests have been performed in order to study the intra-assay reproducibility. The FT was performed according the previous description (experiment 3 and 4) on different lot numbers of membranes. Different samples with concentrations ranging from 1 to 368 mg/L and the standard curve (prepared with highly purified BSA) with standard from 1 to 200 mg/L have been diluted in Tris buffer supplemented with 3% BSA and tested in different replicates on different membranes. The reflectance was measured and the mean, standard deviation and CV's have been calculated. For each experiment the lot numbers of the reagents are indicated. Results are presented in tables 7b to 7g.

- 25 30 The intra-assay reproducibility is lower than 5% and can be as low as 1%.

k) Evaluation of the inter-assays reproducibility

Preliminary experimental conditions

- 35 The FT procedure was performed according previous description (experiment 3 and 4). The standard curve was tested on different lot of membranes, i.e. strips at different dates in order to check the reproducibility of the method (accurate results) independently from the test conditions such as ambient temperature. Results are presented in table 8a.

Experimental conditions additional data

Further to this, extensive tests have been performed in order to study the inter-assay reproducibility. The FT was performed by different users according the previous description
5 (experiment 3 and 4) on different lot numbers of membranes at different days. Different samples with concentrations ranging from 1 to 368 mg/L and the standard curve (prepared with highly purified BSA) with standard from 1 to 200 mg/L have been diluted in Tris buffer supplemented with 3% BSA and tested in different replicates on different membranes. The reflectance was measured and the mean, standard deviation and CV's have been calculated. For each
10 experiment the lot numbers of the reagents are indicated. Results are presented in tables 8b to 8f. CV's are lower than 10 % and can be as low as 1%.

I) Evaluation of another precipitating TMB additional data

- 15 *Aim 1: Another source of precipitating TMB (provided by another company) was tested in order to overcome the lack of discrimination for high values in CRP as shown in table 4b.*

Two different solutions were compared. A TMB 'A' solution was bought from Seramun (15754 Dolgenbrodt, Germany), and compared to a TMB 'B' solution obtained from D-TEK (7000 Mons, Belgium).

20

Experimental conditions

A standard curve has been prepared in Tris diluent buffer and the FT procedure using droplet dispenser has been performed as previously described (experiment 3 and 4).

Results are shown in table 9a. Reflectance units are indicated for each concentration used using
25 the different TMB agents (TMB "A" and "B").

Conclusion

The results showed that TMB "B" induces a better discrimination compared to the TMB "A". Such
30 finding confirmed the variation of quality according source.

Aim 2: In order to guarantee a good reproducibility, a new lot of TMB has been compared with the previous lot in the same test condition on standard curve.

- 35 *Experimental conditions*

2 lots number of TMB have been tested on a purified standard curve ranging from 5 to 100 mg/L and 2 samples on membrane lot 1051.

The variation between 2 lots was calculated by the ratio : $\frac{\text{reflectance reference lot}}{\text{reflectance of the new lot}}$

Results are shown in table 9b.

5 *Conclusion*

The new lot gives comparable results to the previous one (1% variation between both lots).

Aim 3: In order to guarantee a good reproducibility, a new lot of TMB has been compared with the previous lot in the same test condition on samples.

10

The FT was performed according the previous description (experiment 3 and 4) on membrane lot 1065 (produced in June 2001). 2 lots number of TMB have been tested on 2 samples and the diluent buffer only (blank value) in triplicates with a different dispensing device for each of the replicates. The reflectance was measured and the mean, standard deviation and CV's have been calculated. Results are shown in table 9c.

15

Conclusion

The new lot gives comparable results to the previous one within the inter-assays variation.

20

m1). Preliminary determination of the lowest concentration which can be detected.

A standard curve ranging from 2.5 mg/L to 360mg/L was prepared in a Tris diluent buffer containing 3% BSA. The FT procedure was performed according to previous description (experiment 3 and 4).

25

Experimental condition:

Membrane 00 6 4, conjugate anti-CRP HRP labeled with a dilution of 1:700 was used resulting in a final concentration of 0.9 mg/L. 2 commercial TMB were compared: TMB-A from D-TEK (Belgium) and TMB-B from Seramun (Germany). Results are shown in table 10 a. Reflectance units measured for each standard value (mg/L are indicated).

30

Conclusion:

On this lot, the lowest value, i.e. different from 0 which could be detected is 2.5 mg/ml. Values between 180 and 360 mg/L can be discriminated.

35

m2). Additional data related to the lowest concentration which can be detected.

Experimental conditions

A standard curve ranging from 0 mg/L (blank) to 10 mg/L was prepared in a diluent buffer containing 3% BSA. Each concentration has been tested in 4 replicates on different lot of membranes. The FT procedure was performed according to previous description. Mean,
5 standard deviation and CV have been calculated. A statistical analysis (mean \pm 1,5 standard deviation (SD)) determined the lowest value different from zero which can be detected. Results are shown in table 10 b and 10c.

Conclusions

10 The lowest value which can be detected with a reflectance reader is 1mg/L.

4.3 General conclusions

By an accurate measurement of the colored spot, the reflectance reader allow the implementation of quality control procedure and requested specifications. The Intra-assays
15 coefficient of variation may be as low as 1% up to 7% which is totally acceptable for a rapid test. Using the same final dilution of conjugate, the inter-assays coefficient of variation is lower than 10% for the different lot of membranes. By titration of conjugate, lower coefficient of variation can be obtained. A poorly visual discrimination is always confirmed by the reflectance values got with the reader.

20

Example 5. Detection of anti-mitochondrial antibodies (type M2) by the FT method.*5.1 Introduction*

M2 antigen, i.e. pyruvate dehydrogenase is a specific marker for the diagnostic of Primary Biliary Cirrhosis (PBC). The detection of anti-M2 antibodies is a specific tool for the diagnostic of PBC
25 from other hepatic diseases.

5.2 Experiment A:

Membranes type CLN from mdi (Advanced Microdevices) with a pore diameter of 0.45 μ m have been coated with highly purified dehydrogenase complex (Sigma ref. P5194) and blocked
30 according procedures described in headings 2.3 and 2.4. Human anti-M2 antibodies (IgG/IgM) are revealed by the use of anti-human IgG Fab'2 HRP labeled (Dako ref. P406) and anti-human IgM Fab'2 HRP labeled (Dako ref. P322), with concentrations ranging from 0.2 to 0.3 mg/L, followed by the addition of precipitating TMB. Samples have been diluted in 1/100 in a Tris diluent buffer containing 5% BSA and the FT performed as previously described (Experiment 3
35 and 4).

Results

A coating concentration of 0.115 mg/ml corresponding to an enzymatic activity of 0.54 units/ml gave good visual discrimination between negative, low positive (+) and positive result (++). Background (diluent buffer only) was acceptable.

5.2 Experiment B

Membranes type CLN from mdi (Adanced Microdevices with a pore diameter of 0.45µm have been coated with highly purified dehydrogenase complex at a concentration of 0,24 mg/ml (Sigma ref. P5194) and blocked according procedure described in headings 2.3 and 2.4.

Human anti-M2 antibodies (IgG/IgM) are revealed by the use of anti-human IgG Fab'2 HRP labeled (Dako ref. P406) and anti-human IgM Fab'2 HRP labeled (ref. Dako P322) with concentration ranging from 0.2 to 1.0 mg/L, followed by the addition of precipitating TMB. Samples have been diluted 1/50 (as this dilution gave the best discrimination between positive and negative results) in a Tris diluent buffer containing 3% BSA and the FT performed as previously described (experiment 3 and 4). Results have been interpreted by comparison of the intensity of the coloration with the intensity of the cut-off (limit of positivity). Reflectance has also been measured. As there is no correlation between the severity of the disease and the level of anti-M2 antibodies, a "yes/no", i.e. presence of absence of anti-M antibodies is sufficient and no quantitation is needed.

Tested samples featured a positive typical cytoplasmic pattern with the immunofluorescent method. However such immunofluorescent method does not allow to differentiate anti-M2 from other types of anti-mitochondrial antibodies (anti-M4, M5, M9).

Aim of the study

a) Results of the FT were compared with the ELISA method for the detection of anti-M2 antibodies.

ELISA M2 method is an automated method for which the result is obtained within 2 h.

Inter-assays reproducibility also has been studied.

b) inter-assay reproducibility (inter and intra-assay) has been studied.

Results are shown in table 11a and 11b

Interpretation of results:

ELISA : positive result if the Optical Density is > than the OD for the cut-off control.

FT : positive result if the RU is higher then the RU value for the cut-off control.

There is a good correlation (100%) with the ELISA method. No discrepancy in terms of positive versus negative results is observed. The inter-assays reproducibility is lower than 10%.

5

Example 6. Detection of rheumatoid factors by the FT method.

6.1 Introduction.

Rheumatoid factors are human immunoglobulins, i.e. mainly belonging to the IgM isotype directed against human or animal immunoglobulins. Detection of rheumatoid factors is very useful in the diagnosis of Rheumatoid Arthritis.

10

6.2 Experiment

Membranes type CLN from mdi (Advanced Microdevices) with pore diameter of 0.45µm have been coated with purified human immunoglobulins (Sigma ref. 068H4858) and blocked according to procedures described in headings 2.3 and 2.4. Rheumatoid factors type M were revealed by the use of anti-human IgM Fab'2-HRP labeled (Daka ref P322), with a concentration +/- 1.5 mg/L, followed by the addition of precipitating TMB. Samples have been diluted 1/100 in a Tris diluent buffer containing 5% BSA and the FT performed as previously described.

15

6.3 Results

With a coating concentration of 0.53 mg/ml, the visual discrimination between negative samples (<25 IU/ml) and positive result (+/- 200 IU/ml) is acceptable.

20

Example 7: Detection of Salmonella

7.1 Introduction

Detection of possible bacterial food contamination is the aim of the Hazard Analysis Critical Control Point (HACCP) recommendations. *Salmonella* is one of the main bacteria responsible for food mass poisoning. Classical methods such as culture, selection by growth on specific media and biochemical identification are labour and time consuming. Moreover, different bacteria may share same biochemical properties which may induce a wrong identification. Specific antibodies directed against bacterial antigens have now made possible to develop methods for rapid detection of food-borne pathogens. The use of such rapid detection method could allow a faster identification and detection. The aim was to develop a FT for the detection of *Salmonella* sp..

30

7.2 Experimental conditions

Membrane type CLN from mdi (Advanced Microdevices) with a pore diameter of 0.45 μm have been coated with a monoclonal capture antibody purified by A chromatography, specific to *Salmonella typhimurium* (Biodesign ref. C65636M) with a concentration of 0.1 mg/ml. The coating and post-coating procedures are those described in sections 2.3 and 2.4., except that the incubation time for the coating procedure was 5 hours under gentle agitation at RT (18-24°C) and 1 or 2 hour(s) for the post-coating procedure.

In the meantime, a reference *Salmonella typhimurium* sp. (American Type culture collection ref. ATCC 14028 provided by the Laboratory of Microbiology Of the University of Gent) was plated on specific medium (nutrient agar) and incubated in aerobic conditions during 24 hours at 37°C.

a) Testing procedure for identification

For such procedure, a larger test device has been used (test zone of 1 cm) with a larger number of filters -8- (adsorbent pads). 1 pure culture of *Salmonella typhimurium* was diluted in physiologic water. 200 μl of such suspension were transferred to the membrane and let soak. The presence of *Salmonella* was revealed by 200 μl of a second antibody HRP labeled (rabbit anti-salmonella peroxidase conjugated polyvalent for *Salmonella* "O" & "H" antigens (conc. 4 $\mu\text{g/ml}$ Biodesign ref. B65704R) followed by the addition of 200 μl of precipitating TMB. Coloration was stopped by 200 μl of fixative solution. There was a clear blue spot indicating the presence of *Salmonella typhimurium*.

b) Possible quantification

In order to evaluate a possible quantification, a standard curve of *Salmonella typhimurium* ranging from $5,3 \times 10^7$ to $7,0 \times 10^1$ was prepared hypotonic solution and tested with the FT. The different dilutions were counted for quantification. For the performance of the FT procedure, a larger test device has been used, i.e. test zone of 1 cm with a larger number of filters -8- (adsorbent pads). 500 μl of each dilutions were transferred to the membrane and let soak. 2 procedures for sample dispensing were used being 2 X 250 μl of sample, i.e. meaning a first soaking of 250 μl followed by a second deposit of 250 μl of sample and a flush of 500 μl of sample at once.

The presence of *Salmonella* was revealed by 500 μl of a second antibody HRP labeled (rabbit anti-salmonella peroxidase conjugated polyvalent for *Salmonella* "O" & "H" antigens (conc. 4 $\mu\text{g/ml}$ Biodesign ref. B65704R) followed by the addition of 200 μl of precipitating TMB.

Coloration was stopped by 500 µl of fixative solution. Reflectance was measured. Results are shown in table 12.

There is a decrease in the intensity of the blue coloration measured by reflectance units according bacterial concentration meaning that quantification is possible. A flush of 500 µl of sample at once induced a more colored spot.

Example 8: Use of nylon membrane for the detection of CRP protein.

Nylon is a linear synthetic polyamide terminated with either primary amine (positively-charged) or carboxylate (negatively-charged) end groups. Because the nylon membranes possess different surface chemistries from nitrocellulose (which is neutral), the binding properties are different.

Experimental conditions

In order to study the binding capacities of nylon, membranes from the company CUNO have been coated according the procedure described in section 2.3, with 10mmol or 25mmol Tris buffer. Different blocking procedures have been tested, either with BSA (condition A), either with polymer solutions such as polyvinyl alcohol (max. 0.1%) or polyvinyl pyrrolidone (max. 1%) combined with non fat dry milk proteins (condition B). Nylon type C from the company Cuno of 0.45 µ diameter with a lowest ratio of amine to carboxylate end groups resulting in low positive charge have been used. Samples have been tested according procedure described in examples 3 and 4.

Results

By the use of BSA as blocking agent (condition), a colored spot was observed for the positive samples but some significant background was observed for the negative sample. Discrimination between positive and negative results was poor. Membranes blocked with polymer solutions and with non-fat dry milk protein also gave poor results (speckled spot). The first soaking time was too fast and a diffusion phenomenon was observed.

As different binding processes are involved for the binding on nylon membrane, a new optimization of reagents may be performed if such membranes are used. Nevertheless, the general concept as presented in present invention applies.

Example 9: Influence of the molarity in Tris and pH.

The influence of the composition (molarity) and the pH of the applied diluent buffer was studied.

Composition of the diluent buffer varied according conditions A, B and C (see below).

Experimental conditions and procedure

5 Samples have been diluted 1/50 into the diluent A, B and C and have been further tested according example 3 and 4.

Composition of the diluent (for 1 l):

Condition A

10 Lot 1111 A & 1112 : 0.12 g Tris
 0.88 g. NaCl ⇨ pH 7.36
 30 g. BSA

This buffer is adapted to 'whole blood sample' and allows a complete hemolysis of the red cells due to the low concentration of NaCl which induces hypotonic conditions leading to hemolysis. The pH is neutral.

15

Condition B

Lot 1051 : 1.2 g. Tris
 8.8 g NaCl ⇨ pH 9.6
 30 g. BSA

20 This buffer is adapted for serum samples but does not allow a complete hemolysis of red blood cells in case of whole blood is tested. The pH is basic.

Condition C

25 Lot 1113 : 1.2 g Tris
 0.88 g. NaCl ⇨ pH 10.5
 30 g. BSA

This buffer induces hemolysis of the red blood cells but with a basic pH. Results are shown in table 13.

30 There is no statistical difference in the results according buffer composition. However a basic pH increases the flow rate through the membrane. Composition C has been selected.

Reagents

5	A	Membrane : 1062	B	Membrane : 1062	C	Membrane :
		Conjugate : 1083		Conjugate : 1051		Conjugate : 1083
		Diluent : 1111 & 1112		Diluent : 1051		Diluent : 1113
		TMB : 1021		TMB 1021		TMB : 1021
		Fixative : 1081		Fixative : 1081		Fixative : 1081

Example 10: Comparison with another rapid method for the detection of CRP10 *Introduction*

The CRP FT kit from has been benchmarked against the rapid method for the detection of CRP by immunoturbidimetry from the company ORION. The end-user was the emergency unit of a hospital. Samples were coming from patients manifesting signs of viral or bacterial infections. In case of a significant concentration in CRP (> 50 mg/L) the patient was treated with antibiotics.

15 The ORION method is a rapid quantitative method based upon immunoturbidimetry but which requires a reader for the interpretation or results. Such method does not allow visual interpretation of result.

Aim of the study

20 47 samples ranging from negative in CRP concentration to high positive have been tested according Orion kit instructions.

Number of positive samples tested : 17 (36% of the total number of samples)

Number of negative samples tested : 30 (64% of the total number of samples)

25 The FT method from BIO ART has been performed according experiment described in section 3.

Interpretation of results

Results have been interpreted quantitatively with Orion (measurement of the concentration) and semi-quantitatively with BIO ART (comparison of the blue coloration with a reference color chart with the following concentrations 10-25-75 and 200 mg/L). Results are shown in table 14a-14e.

30 There is a good correlation for the negative samples (table 14a). The concordance is 90% (27/30 samples well correlate). 3 samples (12/15/3) are interpreted as low positive with BIO ART and negative with Orion. As no clinical data were available for these 3 samples, no definitive conclusion can be drawn regarding the possibility of either false positive results for BIO ART

35 either false negative results for Orion. For the low positive samples (table 14b), there is a correlation of 92% between both methods (11/12 samples). One sample (31) features a conc. of

25-50 mg/L with BIO ART method and 25 mg/L with Orion. Such difference however, does not induces any misclassification or misdiagnose. The four positive samples (table 14c) are similarly classified either with BIO ART, either with Orion. Only one high positive sample (n°37 see table 14d) has been tested and has a concentration of 200 mg/L with BIO ART and 112 mg/L with Orion. The cut-off level was 8 mg/L for Orion and 10 mg/L for BIO ART meaning that samples with a concentration lower than 8 or 10 mg/L feature normal levels in CRP.

Conclusion:

1. Correlation on the positive samples

There is no false negative result with the BIO ART method (all the samples which are positive with Orion are found positive with the BIO ART method). Correlation on positive samples is thus 100%. Moreover, 16 samples on 17 (except n°31) are classified into the same range of concentration

2. Correlation on negative samples see table 4a)

3 samples (n°12/15/3) are slightly positive with BIO ART and negative with Orion. In absence of clinical data or additional third method, no conclusion can be drawn regarding the possibility of either false positive results (BIO ART method) either false negative result (Orion). Moreover, these 3 low positive results from BIO ART do not induce erroneous diagnosis.

3. General conclusion

Such study indicated that the visual interpretation of results with the BIO ART FT method is valid for the interpretation. The FT from BIO ART features the same technical performances than the other rapid method. However, further to this study, it has been decided to include an additional standard of 1 mg/L in order to improve the visual discrimination between 0 and 10 mg/L

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Tables

Table 1: Main organ and non-organ specific antibodies present in autoimmune diseases.

General classification	
Organ specific antibodies	Non-organ specific antibodies
Anti-parietal cells (eg. Biermer's disease)	Anti-smooth muscle (Eg. chronic active hepatitis CAH type 1)
Anti-thyroid microsomes (eg. Hashimoto's thyroiditis) Grave's thyrotoxicosis	Anti-Liver Kidney microsomes (eg. chronic active hepatitis CAH type 2)
Anti-thyroglobulins (eg. Hashimoto's thyroiditis) Grave's thyrotoxicosis	Anti-mitochondria (Eg. Primary Biliary Cirrhosis)
Anti-Langerhans islets (eg. diabetes)	Anti-reticulin (eg. coeliac disease)
Anti-skin (eg pemphigoid)	Anti-endomysium (Eg. coeliac disease)
Anti-striated muscle (ex. Myastenia gravis)	Anti-nuclear (Eg. System autoimmune diseases: SLA, Sjögren syndrome, scleroderma, dermatomyositis, polymyositis ..)

Table 2: Auto-immune diseases induced by non-organ specific autoantigens

	Diseases	Autoantigen
5	Systemic Lupus Erythematosus	ds-DNA, ss DNA, RNP, Sm, cardiolipin, (SSA)(SSB)
	Drug induced lupus erythematosus	histones
10	Scleroderma	nucleolar, Scl 70
	Crest syndrome	centromere
15	Other rheumatic diseases	ss and/or ds DNA
	Polymyositis	Jo1
	Polymyositis + scleroderma	PM-Scl
20	Rheumatoid arthritis	immunoglobulins (IgG)
	Mixed connective tissue diseases	RNP
25	Sjögren syndrome	SSA/SSB

Table 3: Auto-immune diseases induced by organ specific autoantigens

Disease	non organ specific autoantigens
5 Addison's disease Auto-immune haemolytic anemia Chronic active hepatitis Nuclear laminins Coeliac disease	adrenal cell cytoplasm erythrocytes cell surface lipoproteins, smooth muscles, endomysium
10 Goodpasture's syndrome Graves' thyrotoxicosis Hashimoto's thyroiditis Idiopathic thrombocytopenic purpura Juvenile onset diabetes	basement membrane (glomerular and lung) TSH receptor thyroglobulin platelets islet cell cytoplasm and surface
15 Late onset diabetes Lens induced uveitis Male infertility (some) Multiple sclerosis Myasthenia gravis	insulin receptor lens spermatozoa brain skeletal and heart muscle
20 Pemphigoid Pernicious anemia Primary biliary cirrhosis Primary myxoedema Sympathetic ophthalmia	basement membrane (skin) parietal cell, gastrin receptor, intrinsic factor mitochondria, pyruvate dehydrogenase thyroid uvea
25 Ulcerative colitis Vasculitides Wegener's granulomatosis	colon lipopolysaccharide p ANCA c ANCA

Table 4: Evaluation of the flow-through method as described in the present invention and other existing methods of Nephelometry and Nycomed.

Table 4a: Semi-quantitative method

Ranking	Ident.	Nephelometry	BIO ART FT	NYCOMED	Int.
		[mg/L]	[mg/L]	[mg/L]	
1	114467	0.4	< 11 <11*	<10	Neg.
2	114083	0.6	< 11 <11*	<10	Neg.
3	114196	1.2	< 11 < 11*	<10	Neg.
4	114187	1.7	< 11 < 11*	<10	Neg.
5	114169	1.9	< 11 < 11*	<10	Neg.
6	114058	2.0	< 11 <11*	<10	Neg.
7	114188	2.1	< 11 <11*	<10	Neg.
8	114035	2.5	< 11 < 11*	<10	Neg.
9	114125	2.5	< 11 < 11*	<10	Neg.
10	114086	3.2	< 11 < 11*	< 10	Neg.
11	114206	4.8	< 11 < 11*	=10	Neg./D.
12	114201	5.3	< 11 < 11*	< 10	Neg.
13	114161	6.2	< 11 < 11*	= 10	Neg./D.
14	114197	8.9	< 11 < 11*	slightly > 10	Neg./D.

15	114057	9.3	+/- 20 +/- 15*	slightly > 10	D/D.
16	114011	10.7	lightly > 11 = 11*	darker >10 +/- 20	D/Pos.
17	114085	11.9	lightly > 11 < 11*	darker >10 +/- 20	D/Pos.
18	114118	13.0	= 11 = 11*	25	Pos. +
19	114192	14.2	> 11 but <27.5 > 11 but <27.5*	25	Pos. +
20	114190	17.8	> 11 but <27.5 > 11 but <27.5*	25	Pos. +
21	114084	20.2	+/- 20 [11-27.5]*	slightly > 25 +/- 30	Pos. +
22	114065	24.7	27.5 [11-27.5]*	25-50	Pos. +
23	114015	28.2	27.5 27.5*	25-50	Pos. ++
24	114061	31.7	27.5/ 27.5*	50	Pos. ++
25**	115066	34.9	+/- 69 [50-100] [27.5-69] *	50	Pos. ++
26**	115165	38.5	+/- 69 [50-100] +/- 40*	50	Pos. ++
27	114066	43.0	+/- 69 [50-100] +/-50	darker> 50 but < 100	Pos. ++
28	115166	45.4	+/- 69 [50-100] +/-50	darker> 50 but < 100	Pos. ++
29	114081	47.7	+/- 50 +/-50	+/- 80	Pos. ++
30	115019	52.7	[27.5-69] +/-50	lightly < 100 +/- 90	Pos.++(+)
31	114039	54.7	< 69 [50-100] < 69*	+/- 90	Pos.++(+)

32	114177	60.5	< 69 [50-100] +/- 69*	+/-100	Pos.++(+)
33	115083	61.9	< 69 [50-100] < 69*	100	Pos.++(+)
34*	114016	67.6	+/- 27.5 / +/- 27.5*	100	Pos.++(+)
35**	115026	75.1	lightly > 91 [100-150] lightly < 69 *	lightly > 100	Pos.++(+)
36	114068	84.0	lightly > 91 [100-150] = 91*	100	Pos.++(+)
37**	114179	94.0	= 69 = 91*	lightly > 100	Pos. +++
38	115079	109.0	= 91 = 91*	> 100	Pos. +++
39	115022	122.0	= 91 = 91*	> 100	Pos. +++
40	114073	133.0	[91-137] = 137*	darker > 100 +/-160	Pos.+++
41	114076	157.0	[91-137] = 137*	200	Pos.++++
42	115034	166.0	+/- 137 +/- 137*	200	Pos.++++
43*	114185	181.0	[91-137] [91-137]*	200	Pos.++++
44 **	115042	203.0	= 91 (heterog.spot) = 137*	200	Pos.++++
45	114181	208.0	> 137.5 > 137.5*	200	Pos.++++
46	114019	218.0	slightly < 275 [138-275]	> 200 sat.	Pos.++++
47	115016	231.0	+/- 150 +/-150*	> 200 sat.	Pos.++++

48	114087	258.0	+/-150 +/-150*	> 200 sat.	Pos.++++
49	115064	312.0	+/- 275 +/-275*	> 200 sat.	Pos.++++
50	115084	368.0	+/- 275 +/-275*	> 200 sat.	Pos.++++

Table 4b: Feasibility study of a quantitative method*4.b.1. Standard curve*

Standards mg/L	Reflectance Red filter BIO ART	Reflectance Red Filter Nycomed
275	77.2	69.9
120	84.3	92.3
60	95.4	112.5
40	101.2	131.4
20	137.0	152.1
10	153.6	161.5

5 *4.b.2. Samples*

Sample	Nephelometry mg/L announced conc.	Reflectance Red filter BIO ART	Conc. mg/L	Reflectance Red filter Nycomed	Conc. mg/L
1	0.4	168.4	<10	190.4	< 10
2	0.6	169.1	<10	192.4	< 10
3	1.2	161.3	<10	np	
4	1.7	171.9	<10	190.5	<10
5	1.9	171.3	<10	np	
6	2.0	175.4	<10	189.1	<10
7	2.1	158.3	<10	np	
8	2.5	164.2	<10	190.2	<10
9	2.5	150.1	< 5	np	
10	3.2	157.4	<10	190.5	<10
11	4.8	164.8	<10	np	
12	5.3	162.0	<10	186.7	<10
13	6.2	173.8	<10	np	
14	8.9	155.9	< 5	174.0	<10
15	9.3	153.8	9	np	
16	10.7	155.2	< 5	170.1	10

17	11.9	151.2	11	np	
18	13.0	148.0	12	164.7	11
19	14.2	130.2	20	np	
20	17.8	140.7	15	154.3	16
21	20.2	132.9	19	np	
22	24.7	127.6	23	146.2	24
23	28.2	109.6	32	np	
24	31.7	123.6	24	140.5	28
25	34.9	110.6	33	np	
26	38.5	114.9	30	131.4	36
27	43.0	106.8	38	np	
28	45.4	99.7	40	121.9	52
29	47.7	94.7	50	np	
30	52.7	104.1	37	117.1	56
31	54.7	142.6	15?	119.9	50
32	60.5	98.2	60	114.1	64
33	61.9	101.4	52	111.2	70
34	67.6	100.8	56	113.1	68
35	75.1	98.9	60	110.2	72
36	84.0	102.3	52	103.0	92
37	94.0	94.9	78	100.4	100
38	109.0	94.6	78	87.4	160
39	122.0	97.6	64?	88.5	150
40	133.0	83.9	140	81.8	190
41	157.0	84.9	135	80.9	200
42	166.0	85.5	120	83.8	175
43	181.0	84.4	136	75.0	240
44	203.0	101.9	52 ?	74.0	260
45	208.0	83.1	150 ?	72.6	250
46	218.0	101.6	52?	68.8	300
47	231.0	99.1	54?	68.9	300
48	258.0	106.4	58?	71.6	280
49	312.0	91.3	91?	70.9	280
50	368.0	88.6	100 ?	60.5	400

Table 4 C: Feasibility of a quantitative method**5 BIO ART standard curve :**

<i>standard</i>	<i>Ru's</i>
<i>mg/L</i>	
0	189
1	183
10	151
25	132
75	108
200	74

Curve :

$$y = -0,8465x + 3,0706$$

10 $R^2 = 0,991$

Nycomed standard curve :

<i>standard</i>	<i>Ru's</i>
<i>mg/L</i>	
0	170
10	162
40	131
60	113
120	92
275	70

15 Curve :

$$y = -1,2818x + 5,4987$$

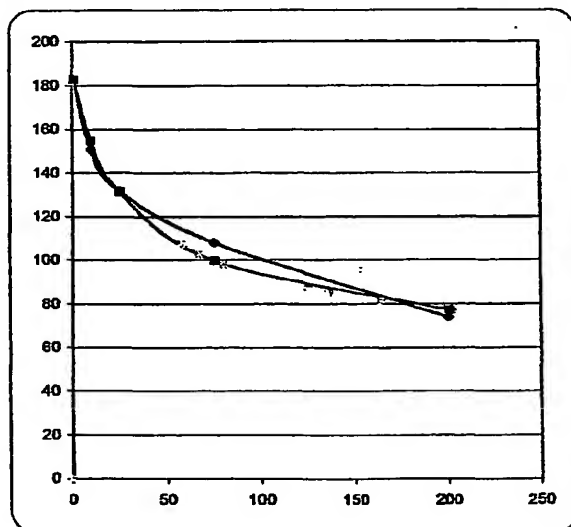
$$R^2 = 0,995$$

sample	Nephelometry Ann.conc.mg/L	BIO ART RU's	BIO ART Conc.mg/L	NYCOMED RU's	NYCOMED Conc. mg/L
0 – 5 mg/L					
1	0,4	176	2,6	190	<10
2	0,6	176	2,6	192	<10
3	1,2	178	2,1	np	
4	1,7	169	4,6	190	<10
5	1,9	164	6,3 *	np	
6	2,0	173	3,4	189	<10
7	2,1	176	2,6	np	
8	2,5	180	1,6	190	<10
9	2,5	166	5,6 *	np	
10	3,2	170	4,3	191	<10
11	4,8	156	9,5 *	np	
5,1 -10 mg/L					
12	5,3	152	11,4	187	<10
13	6,2	147	14,0	np	
14	8,9	156	9,5	174	<10
15	9,3	158	8,6	np	
10,1- 25 mg/L					
16	10,7	148	13,4	170	10
17	11,9	141	17,7	np	
18	13,0	141	17,7	165	6,3
19	14,2	151	11,9	np	
20	17,8	133	23,6	154	17
21	20,2	133	23,6	np	
22	24,7	148	13,4	146	24,8
25,1 – 75 mg/L					
23	28,2	132	24,5	np	
24	31,7	127	29,1	140	31,0
25	34,9	127	29,1	np	
26	38,5	112	48,6	131	41,3

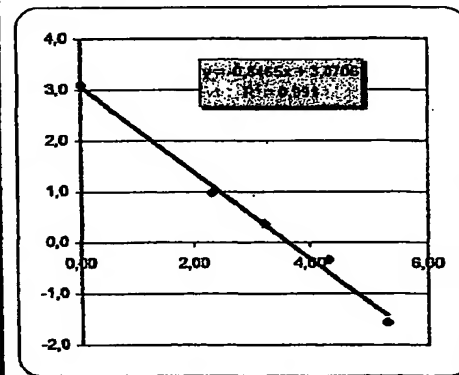
27	43,0	131	25,3	np	
28	45,4	123	33,4	122	53,3
29	47,7	124	32,3	np	
30	52,7	112	48,6	117	60,9
31	54,7	115	43,8	np	
32	60,5	117	40,9	120	56,2
33	61,9	112	48,6	np	
34	67,6	106	59,9	113	67,6
75,1 – 200 mg/L					
35	75,1	111	50,3	110	73,1
36	84	104	64,3	103	87,8
37	94	107	57,8	100	95,1
38	109	98	80,1	87	137,3
39 **	122	103	66,6	89	129,3
40	133	112	48,6	82	161,0
41	157	85	136,2	81	166,5
42	166	81	164,3	84	150,8
43	181	77	201,9	75	207,2

[illegible]

STD (x)	lnx	interpol.	Ecart%	Courbe moyenne	Y Moy.	Probil	Logit	Extrapol.	Measure (y)
		0	0	0	-38			0	
1	0,000	1,0	-3,3	183	98	22,167	3,099	183	183
10	2,303	11,9	18,5	151	73	2,658	0,978	155	161
25	3,219	24,5	-2,1	132	59	1,439	0,364	131	132
75	4,317	55,8	-25,8	108	42	0,716	-0,334	100	108
200	5,298	239,4	19,7	74	17	0,209	-1,567	77	74



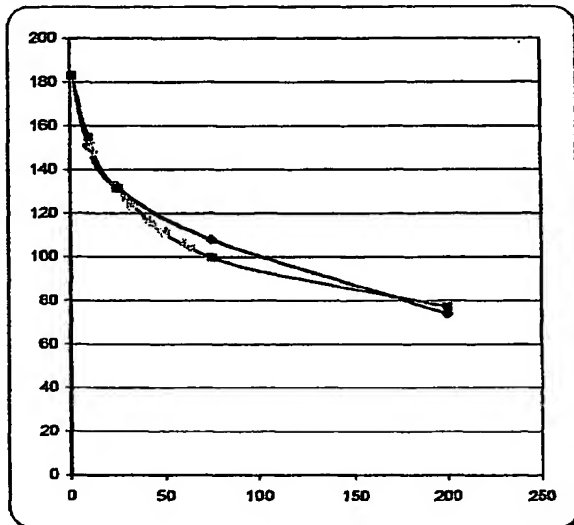
Mean	189
NS	50
50% Int.	37,8
S	-0,847
lnA	3,07061
r2	0,99101

[illegible]

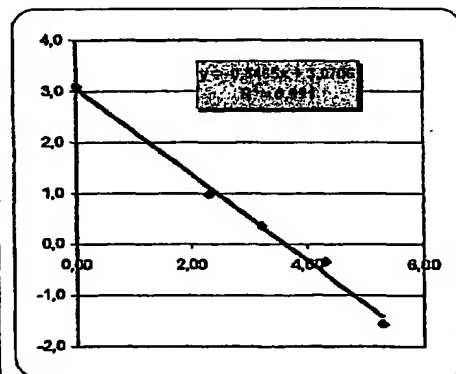
Assay protocol

comparative study (table 4c)	
lot 1062	

STD (x)	lnx	Interpol.	Ecart%	Courbe moyenne	Y Moy.	Probit	Logit	Extrapol.	Measure (y)
1	0,000	0	0	183	96	22,167	3,099	183	183
10	2,303	11,9	-3,3	151	73	2,658	0,878	155	151
25	3,219	24,5	-2,1	132	59	1,439	0,384	131	132
75	4,317	55,8	-25,6	108	42	0,716	-0,334	100	108
200	5,298	239,4	19,7	74	17	0,209	-1,567	77	74



Mean	183
NS	50
50% int.	57,8
S	-0,847
lnA	3,07061
r2	0,99101

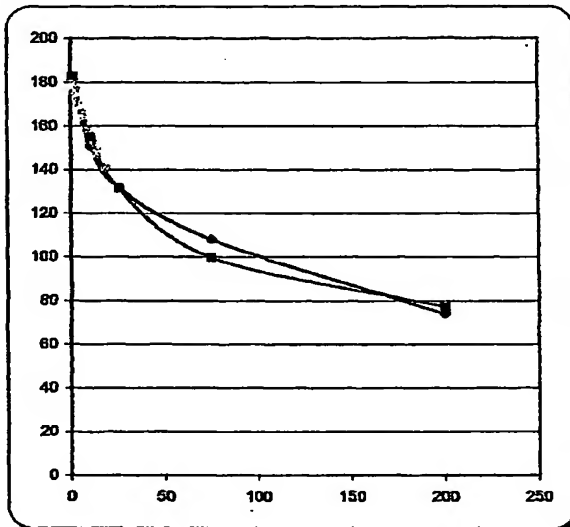


Snp	Y	Probit	Valeur
19	151	2,658	11,9
20	133	1,482	23,6
21	133	1,482	23,6
22	148	2,390	13,4
23	132	1,439	24,6
24	127	1,242	29,1
25	127	1,242	29,1
26	112	0,805	48,6
27	131	1,397	25,3
28	123	1,106	33,4
29	124	1,138	32,3
30	112	0,805	48,6
31	115	0,878	43,8
32	117	0,931	40,9
33	112	0,805	48,6
34	106	0,676	69,9
35	111	0,782	60,3
36	104	0,635	64,3

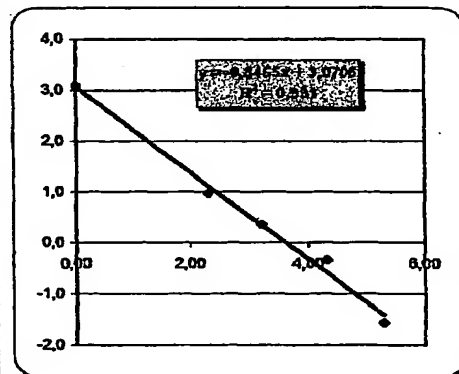
Assay protocol

comparative study (Table 4c)	
lot 1062	

STD (x)	lnx	Interpol.	Ecart%	Courbe moyenne	Y Moy.	Probit	Logit	Extrapol.	Measure (y)
1	0,000	0	0	0	-36			0	
10	2,303	11,9	-3,3	183	96	22,187	3,099	183	183
25	3,219	24,5	18,5	151	73	2,658	0,978	155	151
75	4,317	55,8	-2,1	132	59	1,439	0,384	131	132
100	4,605	69,7	-25,6	108	42	0,716	-0,334	100	108
200	5,298	239,4	19,7	74	17	0,209	-1,567	77	74



Mean	189
NS	50
50% Int.	37,6
S	-0,847
lnA	3,07051
r2	0,99101



Smp	Y	Probit	Valeur
-----	---	--------	--------

1	176	9,692	2,6
2	176	9,692	2,6
3	178	11,638	2,1
4	169	5,950	4,6
5	164	4,560	6,3
6	173	7,688	3,4
7	178	9,692	2,6
8	180	14,444	1,6
9	166	5,043	5,6
10	170	6,318	4,3
11	158	3,212	9,6
12	152	2,757	11,4
13	147	2,310	14,0
14	155	3,212	9,6
15	158	3,484	8,6
16	148	2,390	13,4
17	141	1,896	17,7
18	141	1,896	17,7

Table 5: Evaluation of different membrane lots.

Standard concentration mg/L	Membrane lot 00102	Membrane lot 00103	Membrane lot 00106	Membrane lot 00107
<i>dimensions</i>	<i>8.5 cm X 17 cm</i>	<i>0.8 cm X 17 cm</i>	<i>0.8 cm X 17.5 cm</i>	<i>8.5 cm X 17 cm</i>
275	65.2	67.5	62.9	68.6
137.5	65.4	75.8	76.3	76.6
91	93.7	78.7	88.1	90.9
69	88.6	84.8	90.6	86.9
27.5	122.4	113.5	116.5	120.7
11	119.9	136.7	144.0	113.6

Table 6: Testing of different lots of membranes

Concentration mg/L	Membrane lot 0092	Membrane Lot 0093	Membrane Lot 0094	Membrane Lot 00102	Membrane Lot 00104
<i>strips</i>	<i>8.5 cm X 17.5 cm</i>	<i>8.5 cm X 17.5 cm</i>	<i>8.5 cm X 17.5 cm</i>	<i>8.5 cm X 17.5 cm</i>	<i>8.5 cm X 17.5 cm</i>
208	78.7	80.7	81.0	76.1	73.1
157	77.8	93.7	90.0	83.6	82.7
94	79.5	100.3	98.8	96.9	87.1
48	106.1	107.7	107.8	93.5	109.1
20	126.8	131.5	138.2	104.4/121.7	132.6
15	np	146.7	147.6	117.7	137.3
QC	QC rejected	QC passed	QC passed	QC rejected	QC passed

Table 7: Evaluation of intra-assay reproducibility**Table 7 a**

CRP concentration mg/L	Reflectance lot 00106	Reflectance lot 0064	Reflectance lot 00111	Reflectance lot 00112
157	96.9	80.7	92.2	72.6
	93.9	82.8	79.2	91.2
	100.9	81.5	81.7	84.1
	109.4	80.4	89.4	84.6
<i>Mean</i>	<i>100.3</i>	<i>81.3</i>	<i>85.6</i>	<i>83.1</i>
<i>Standard deviation</i>	<i>6.7</i>	<i>1.2</i>	<i>6.15</i>	<i>7.7</i>
<i>Coefficient of variation</i>	<i>6.6 %</i>	<i>1.5 %</i>	<i>7.2 %</i>	<i>9.3 %</i>
47.8	108.5	103.6	93.3	101.5
	122.2	104.3	101.4	98.8
	131.4	101.1	106.8	100.6
	120.9	101.0	109.4	97.1
<i>Mean</i>	<i>120.8</i>	<i>102.2</i>	<i>102.7</i>	<i>99.5</i>
<i>Standard deviation</i>	<i>9.4</i>	<i>2.0</i>	<i>7.1</i>	<i>2.0</i>
<i>Coefficient of variation</i>	<i>7.7 %</i>	<i>1.9 %</i>	<i>6.9 %</i>	<i>2.0 %</i>
9	168.2	153.2	133.6	144.9
	156.5	154.4	139.4	152.3
	171.0	155.1	139.1	140.8
	168.5	154.5	136.4	145.7
<i>Mean</i>	<i>166.0</i>	<i>154.3</i>	<i>137.1</i>	<i>144.7</i>
<i>Standard deviation</i>	<i>6.5</i>	<i>0.8</i>	<i>2.7</i>	<i>5.4</i>
<i>Coefficient of variation</i>	<i>3.9 %</i>	<i>0.5 %</i>	<i>2.0 %</i>	<i>3.7 %</i>

Table 7: Evaluation of Intra-assay reproducibility**Table 7 b**

Reagents (lot n°)	
Membrane	1062
Conjugate	1083
Diluent buffer	1111 A
TMB	1021
Fixative	1081

sample	RU's	sample	RU's
03 (1mg/L)	175	10 (3 mg/L)	158
	166		163
	167		167
	175		162
Mean	170	Mean	162
SD	4,9	SD	4
%CV	2,8	%CV	2,5
15 (9 mg/L)	152	21 (20 mg/L)	142
	155		128
	158		134
	165		137
Mean	157	Mean	134
SD	5,2	SD	5,5
%CV	3,3	%CV	4,1
29 (48 mg/L)	123	36 (84 mg/L)	101
	111		103
	128		102
	115		101
Mean	118	Mean	101
SD	6,7	SD	0,9
%CV	5,7	%CV	1,0
38 (109 mg/L)	100	50 (368 mg/L)	80
	95		79
	90		81
	96		77
Mean	94	Mean	79
SD	3,7	SD	2,1
%CV	3,9	%CV	2,7

Table 7: Evaluation of intra-assay reproducibility**Table 7c**

5	Reagents (lot n°)
	Membrane 1063
	Conjugate 1083
	Diluent buffer 1081
	TMB 1021
10	Fixative 1081

sample	RU's	sample	RU's
02 (0,6 mg/L)	178	17(12 mg/L)	134
	180		147
	169		146
	168		144
	170		152
	176		146
Mean	174		145
SD	5,2		5,9
%CV	2,9		4,1
22 (24,7 mg/L)	118	35 (75 mg/L)	103
	122		99
	120		105
	113		103
	125		100
	126		98
Mean	121	Mean	102
SD	4,6	SD	2,7
%CV	3,8	%CV	2,6
44 (203 mg/L)	77		
	81		
	73		
	72		
	75		
	70		
Mean	75		
SD	3,9		
%CV	5,3		

Table 7: Evaluation of intra-assay reproducibility**Table 7d**

5	Reagents	
	Membrane	1062
	Conjugate	1083
	Diluent buffer	1081
	TMB	1021
10	Fixative	1081

sample	RU's	sample	RU's
02 (0,6 mg/L)	178	17(12 mg/L)	134
	180		147
	169		146
	168		144
	170		152
	176		146
Mean	174	Mean	145
SD	5,2	SD	5,9
%CV	2,9	%CV	4,1
22 (24,7 mg/L)	118	35 (75 mg/L)	103
	122		99
	120		105
	113		103
	125		100
	126		98
Mean	121	Mean	102
SD	4,6	SD	2,7
%CV	3,8	%CV	2,6
44 (203 mg/L)	77		
	81		
	73		
	72		
	75		
	70		
Mean	75		
SD	3,9		
%CV	5,3		

Table 7: Evaluation of intra-assay reproducibility**Table 7e**

5	Reagents	
	Membrane	1061 b
	Conjugate	1083
	Diluent buffer	1081
	TMB	1021
10	Fixative	1081

sample	RU's	sample	RU's
02 (0,6 mg/L)	169	17 (12 mg/L)	127
	167		132
	168		140
	165		139
	169		127
	170		140
Mean	168		134
SD	2,1		6,8
%CV	1,2		4,7
22 (32 mg/L)	111	35 (75 mg/L)	99
	110		95
	112		98
	113		100
	112		98
	111		100
Mean	111	Mean	98
SD	0,8	SD	1,9
%CV	0,7	%CV	1,9
44 (203 mg/L)	84		
	80		
	80		
	85		
	88		
	81		
Mean	83		
SD	3,2		
%CV	3,9		

Table 7: Evaluation of Intra-assay reproducibility**Table 7f**

5	Reagents	
	Membrane	1066
	Conjugate	1083
	Diluent buffer	1081
	TMB	1021
10	Fixative	1081

sample	RU's	sample	RU's
02 (0,6 mg/L)	176	17(12 mg/L)	139
	166		132
	164		138
	163		130
Mean	167	Mean	135
SD	6,0	SD	4,4
%CV	3,6	%CV	3,3
21 (20mg/L)	119	36 (84 mg/L)	93
	119		101
	114		96
	114		96
Mean	116	Mean	96
SD	2,9	SD	3,3
%CV	2,5	%CV	3,4
50 (368 mg/L)	75		
	77		
	83		
	86		
Mean	80		
SD	5,1		
%CV	6,3		

Table 7: Evaluation of intra-assay reproducibility**Table 7g**

5

10

Reagents	
Membrane	1071
Conjugate	1083
Diluent buffer	1081
TMB	1021
Fixative	1081

sample	RU's	sample	RU's
02 (0,6 mg/L)	171	17(12 mg/L)	125
	174		139
	178		132
	167		132
Mean	172	Mean	132
SD	4,7	SD	5,7
%CV	2,7	%CV	4,3
21 (20 mg/L)	111	36 (84 mg/L)	85
	117		100
	106		79
	107		89
Mean	110	Mean	88
SD	5,0	SD	8,8
%CV	4,5	%CV	10,0
50 (368 mg/L)	67		
	73		
	63		
	67		
Mean	68		
SD	4,1		
%CV	6,1		

Table 7: Evaluation of intra-assay reproducibility**Table 7h**

5	Reagents	
	Membrane	1062
	Conjugate	1083
	Diluent buffer	1113
	TMB	1021
10	Fixative	1081

sample	RU's	sample	RU's
03 (1,2 mg/L)	182	10 (3,2 mg/L)	165
	182		164
	177		172
	178		165
Mean	180		167
SD	2,6		3,7
%CV	1,5		2,2
21 (20 mg/L)	123	29 (48 mg/L)	110
	136		103
	143		108
	130		97
Mean	132	Mean	104,5
SD	9,7	SD	5,8
%CV	7,4	%CV	5,5
36 (84 mg/L)	89	38 (109 mg/L)	89
	95		93
	94		81
	101		82
Mean	95	Mean	86
SD	4,9	SD	5,7
%CV	5,2	%CV	6,7

Table 8: Evaluation of the inter-assay reproducibility**Table 8a:**

<i>Std conc.</i> <i>mg/L</i>							<i>Mean</i>	<i>SD</i>	<i>CV %</i>
Lot	00106	00106	00111	00112	00114	00111			
275	62.9	58.4	68.7	68.2	75.4	70.1	67.3	5.9	8.7
137.5	76.3	75.7	73.0	78.2	82.6	77.8	77.3	3.2	4.1
91	88.1	78.8	85.6	89.2	84.6	80.0	84.4	4.2	5.0
69	90.6	89.6	99.5	95.1	93.6	86.6	92.5	4.6	4.9
27.5	116.5	102.4	115.3	108.6	112.9	102.4	109.7	6.2	5.7
11	114.1	123.2	137.1	130.9	134.9	122.9	127.2	8.7	6.8

Table 8b: Evaluation of the inter-assay reproducibility.

Reagents	
Membrane	1062
Conjugate	1083
Diluent buffer	1081 & 1111A
TMB	1021
Fixative	1081

10

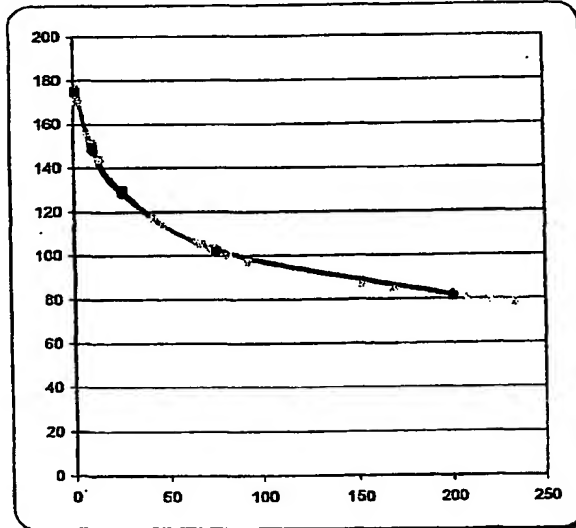
sample	RU's	standard	RU's
02 (0,6 mg/L)	174	1mg/L	173
	178		172
	174		172
	176		182
Mean	176	Mean	175
SD	2	SD	4,9
%CV	1,1	%CV	2,8
17 (12 mg/L)	137	10mg/L	147
	150		154
	147		141
	141		150
Mean	144	Mean	148
SD	5,8	SD	5,5
%CV	4,1	%CV	3,7
22 (24,7 mg/L)	118	25 mg/L	125
	113		135
	120		121
	/		131
Mean	117	Mean	128
SD	3,6	SD	6,2
%CV	3,1	%CV	4,9
35 (75 mg/L)	106	75 mg/L	104
	104		103
	101		97
	111		108
Mean	106	Mean	103
SD	4,2	SD	4,5
%CV	4,0	%CV	4,4
44 (203 mg/L)	76	200 mg/L	89
	78		90
	74		74
	85		73
Mean	78	Mean	82
SD	4,8	SD	9,2
%CV	6,2	%CV	11,3
		blanc	180

			181
			181
			181
		Mean	181
		SD	0,5
		%CV	0,3

Assay protocol

inter-assays table 8b	
standard curve lot 1062	

STD (x)	lnx	Interpol.	Ecart%	Courbe moyenne	Y Moy.	Probit	Logit	Extrapol.	Measure (y)
1	0,000	0	0	0	-38	20,833	3,037	0	175
10	2,303	11,1	10,8	148	75	2,970	1,088	150	148
25	3,219	27,1	8,3	128	60	1,472	0,386	130	128
75	4,317	72,8	-3,2	103	40	0,679	-0,388	102	103
200	5,298	187,3	-6,4	82	24	0,323	-1,129	81	82



Mean	181
NS	60
50% Int.	44,3
S	-0,784
lnA	2,97213
r2	0,99827

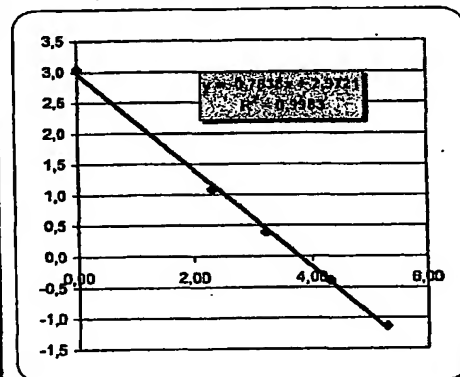


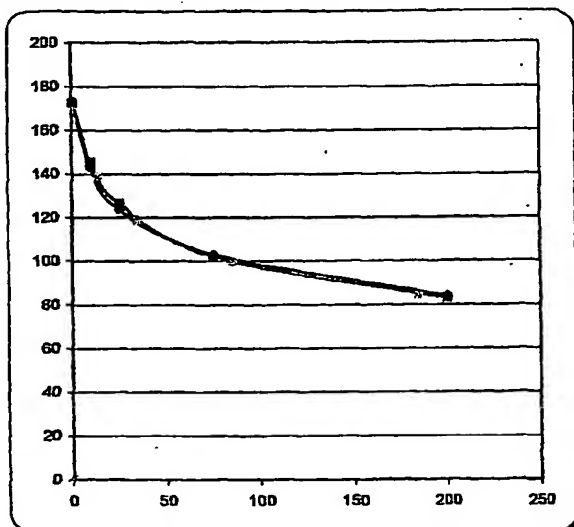
Table 8c: Evaluation of the inter-assay reproducibility

5	Reagents		
	Membrane 1063		
	Conjugate 1083		
	Diluent buffer 1081 & 1111A		
	TMB 1021		
	Fixative 1081		
	sample	RU's	standard
	02 (0,6 mg/L)	169	1mg/L
		170	
		173	
		173	
	Mean	171	Mean
	SD	2,1	SD
	%CV	1,2	%CV
	17 (12 mg/L)	132	10mg/L
		143	
		141	
		Np	
	Mean	139	Mean
	SD	5,9	SD
	%CV	4,2	%CV
	22 (24,7 mg/L)	113	25 mg/L
		122	
		122	
		118	
	Mean	119	Mean
	SD	4,3	SD
	%CV	3,6	%CV
	35 (75 mg/L)	94	75 mg/L
		111	
		101	
		91	
	Mean	99	Mean
	SD	8,9	SD
	%CV	8,9	%CV
	44 (203 mg/L)	75	200 mg/L
		96	
		83	
		82	
	Mean	84	Mean
	SD	8,8	SD
	%CV	10,4	%CV
			blanc
			183
			179

Assay protocol

Inter-assays table 8c	
standard curve lot 1083	

STD (x)	lnx	Interpol.	Ecart%	Courbe moyenne	Y Moy.	Probit	Logit	Extrapol.	Measure (y)
1	0,000	0	0	0	-38	13,667	2,615	0	173
10	2,303	11,3	13,1	173	93	2,385	0,869	172	143
25	3,219	28,0	12,0	143	70	1,276	0,244	127	124
75	4,317	71,1	-5,2	124	56	0,671	-0,399	102	103
200	5,298	184,8	-7,8	103	40	0,347	-1,059	83	84



Mean	182
NS	50
50% Int.	39,9
S	-0,680
lnA	2,54298
r2	0,99711

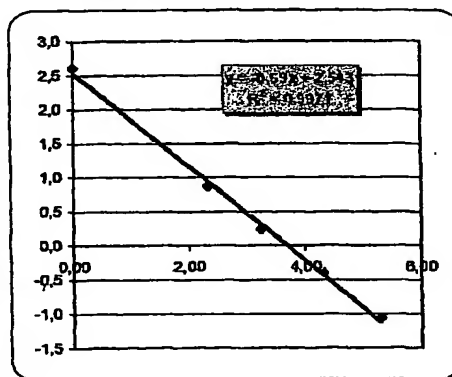


Table 8 d: Evaluation of the inter-assay reproducibility

5	Reagents
	Membrane 1066
	Conjugate : 1083 & 1081
	Diluent buffer : 1081 & 1111A
	TMB : 1021
10	Fixative : 1081

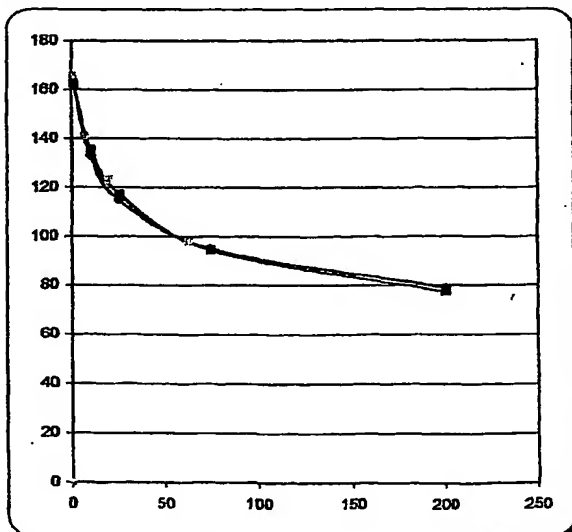
standard	RU's
200 mg/L	83
	76
	81
	95
	78
	74
	67
Mean	79
SD	8,1
%CV	10,2
75 mg/L	104
	93
	92
	104
	95
	92
	87
Mean	95
SD	6,4
%CV	6,4
25 mg/L	125
	119
	109
	126
	110
	110
	108
Mean	115
SD	7,9
%CV	6,8
10 mg/L	130
	130
	127
	131
	135
	132
	148
Mean	133
SD	6,9

%CV	5,2
1 mg/L	159
	160
	162
	168
	161
	167
	163
Mean	163
SD	3,4
%CV	2,1

Assay protocol

reproducibility (Table 2d)	
lot 1066	

STD (x)	lnx	Interpol.	Ecart%	Courbe moyenne	Y Moy.	Probit	Logit	Extrapol.	Measure (y)
1	0,000	0	0	0	41	12,556	2,530	0	163
10	2,303	11,4	14,2	133	68	2,128	0,755	135	133
25	3,219	28,0	11,9	115	53	1,140	0,131	117	115
75	4,317	73,1	-2,8	95	37	0,584	-0,537	94	95
200	5,288	180,1	-9,9	79	24	0,312	-1,165	77	79



Mean	172
NS	60
50% Int.	23,8
S	-0,696
lnA	2,45064
r2	0,9967

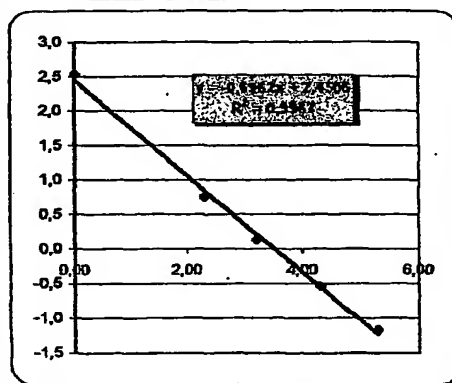


Table 8 e: Evaluation of the inter-assay reproducibility

5

Reagents
Membrane 1066
Conjugate : 1083 & 1081
Diluent buffer : 1081 & 1111A
TMB : 1021
Fixative : 1081

sample	RU's
44 (203 mg/L)	76
	70
	72
	80
	78
Mean	75
SD	4,1
%CV	5,5
35 (75 mg/L)	102
	90
	91
	101
	104
Mean	98
SD	6,6
%CV	6,7
22 (24,7mg/L)	118
	115
	120
	129
	132
Mean	123
SD	7,3
%CV	6,0
17 (12 mg/L)	133
	129
	133
	154
	157
Mean	141
SD	13,1
%CV	9,3
2 (0,6 mg/L)	162
	162
	169
	171
	166
Mean	166
SD	4,0
%CV	2,4

Table 8 f: Evaluation of the inter-assay reproducibility

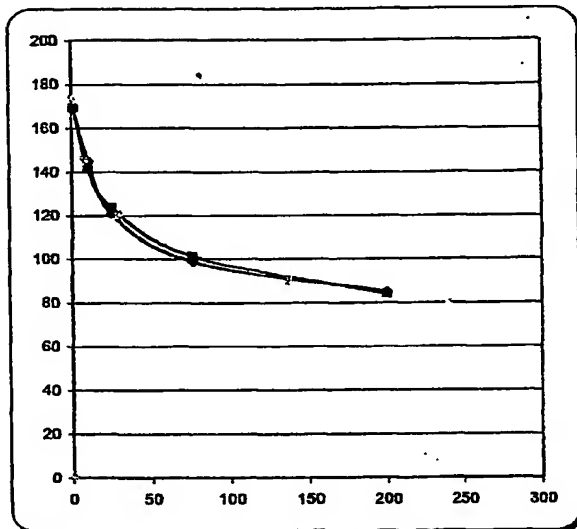
5	Reagents	
	Membrane	1065
	Conjugate	1083
	Diluent buffer	1081 & 1111A
	TMB	1021
	Fixative	1081

sample	RU's	standard	RU's
02 (0,6 mg/L)	173	1mg/L	164
	172		164
	175		171
	175		177
Mean	174	Mean	169
SD	1,5	SD	6,3
%CV	0,9	%CV	3,7
17 (12 mg/L)	155	10mg/L	147
	145		148
	149		140
	136		143
Mean	146	Mean	145
SD	8,0	SD	3,7
%CV	5,5	%CV	2,6
22 (24,7 mg/L)	115	25 mg/L	110
	119		136
	121		119
	124		119
Mean	120	Mean	121
SD	3,8	SD	10,8
%CV	3,2	%CV	8,9
35 (75 mg/L)	92	75 mg/L	98
	84		98
	97		92
	88		106
Mean	90	Mean	99
SD	5,6	SD	5,7
%CV	6,2	%CV	5,8
44 (203 mg/L)	87	200 mg/L	84
	75		84
	80		83
	81		87
Mean	81	Mean	85
SD	4,9	SD	1,7
%CV	6,1	%CV	2,0

Assay protocol

Inter-assays table 8f	
standard curve lot 1065	

STD (x)	lnx	Interpol.	Ecart%	Courbe moyenne	Y Moy.	Probit	Logit	Extrapol.	Measure (y)
1	0,000	0	0	0	-38	9,154	2,214	169	169
10	2,303	1,1	5,3	189	90	2,568	0,943	141	146
25	3,219	28,5	-19,7	145	72	1,164	0,152	124	121
76	4,317	84,2	12,3	99	37	0,590	-0,527	101	99
200	5,298	185,0	-7,5	85	27	0,381	-1,019	84	85



Mean	182
NS	60
50% Int.	36,3
S	-0,628
lnA	2,24843
r2	0,89478

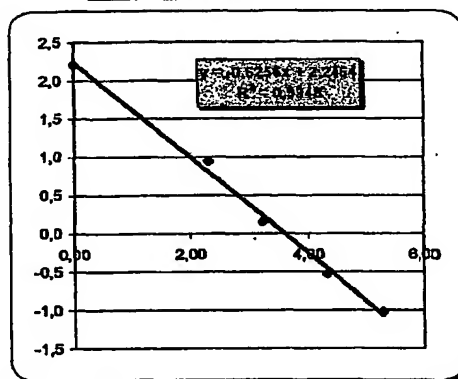


Table 9: Evaluation of different TMB agents5 **Table 9a:** Evaluation of different TMB agents (aim 1):

CRP Concentration mg/L	Reflectance with TMB "A"	Reflectance with TMB "B"
275	77.2	59.0
137.5	84.3	67.5
91	95.4	73.9
69	101.2	89.0
27.5	136.9	105.2
11	153.6	123.8

Table 9 b: Evaluation of 2 different lots of TMB (aim 2).

5	Reagents	
	Membrane	1051
	Conjugate	1051
	Diluent buffer	1051
	Fixative	1021

<i>Standard</i>	<i>TMB lot 207 (ref.)</i>	<i>TMB lot 629</i>	<i>% ref.</i>
mg/L	Ru's	Ru's	Ratio 207/629
100	91	87	95,6
50	103	118	114,5
10	144	141	97,9
5	148	149	100,6
+ ctrl Nycomed	137	121	88,3
+ sample dil. 1/200	114	127	111,4
			Mean : 101,4 +1,04%

Table 9c: Evaluation of 2 different lot of TMB on samples

5	Reagents	
	Membrane	1065
	Conjugate	1083
	Diluent buffer	111A
	Fixative	1081

Samples	TMB lot 629 (ref)	TMB lot 917	% diff.
	RU's	RU's	
29 (48 mg/L)	113	125	
	127	117	
	113	132	
mean	118	125	5,4 %
std dev.	8,0	7,5	
CV%	6,9	6,0	
50 (368 mg/L)	58	58	
	76	62	
	70	70	
mean	69	63	9,5%
std dev.	10	6,1	
CV%	14,6	9,6	
Blank (0 mg/L)	185	176	
	178	175	
	179	175	
mean	181	175	3,4 %
std dev.	3,8	0,6	
CV%	2,1	0,3	
		Mean	6,1%

Table 10: Determination of the lowest detection limit.**Table 10 a:**

5	Reagents (lot n°)	
	Membrane	1061b
	Conjugate	1083
	Diluent buffer	1111
	TMB	1021
	Fixative	1081

10

Standard concentration	RU's
0 mg/l	184
	191
	181
	189
mean	186,25
Std dev.(SD)	4,57
CV%	2,45
1,5 SD	6,85
Mean +/- 1.5 SD	179,4 – 193,1
2 SD	9,4
Mean +/- 2SD	176,9 – 195,7
1 mg/L	160
	162
	172
	159
mean	163,25
Std dev.(SD)	5,97
CV%	3,65
1,5 SD	8,94
Mean +/- 1.5 SD	154,3 – 172,2
2 SD	11,9
Mean +/- 2SD	151,4 – 175,5
10 mg/L	143
	138
	133
	144
mean	139,5
Std dev.(SD)	5,1
C V%	3,6
1,5 SD	7,6
Mean +/- 1.5 SD	131,9 – 147,1
2 SD	10,1
Mean +/- 2SD	129,4 – 149,6

Table 10 b: Determination of the lowest detection limit.

5

Reagents (lot n°)	
Membrane	1106
Conjugate	1083
Diluent buffer	1081
TMB	1021
Fixative	1081

Standard concentration	RU's
0 mg/l	178
	183
mean	180,5
Std dev.(SD)	3,53
CV%	1,95
1,5 SD	5,3
Mean +/- 1.5 SD	175,2 – 185,8
2 SD	7,1
Mean +/- 2SD	173,4 – 187,6
1 mg/L	169
	173
mean	171,0
Std dev.(SD)	2,82
CV%	1,6
1,5 SD	4,2
Mean +/- 1.5 SD	166,8 – 175,2
2 SD	5,6
Mean +/- 2SD	165,4 – 176,6
10 mg/L	145
	136
mean	140,05
Std dev.(SD)	6,4
C V%	4,5
1,5 SD	9,5
Mean +/- 1.5 SD	131,0 – 150,0
2 SD	12,7
Mean +/- 2SD	127,8 – 153,2

Table 10 c: Determination of the lowest detection limit.

5

10

Reagents (lot n°)	
Membrane	1071
Conjugate	1083
Diluent buffer	1081
TMB	1021
Fixative	1081

Standard concentration	RU's
0 mg/l	171
	177
mean	174
Std dev.(SD)	4,2
CV%	2,4
1,5 SD	6,4
Mean +/- 1.5 SD	167,6 – 180,4
2 SD	8,4
Mean +/- 2SD	165,6 – 182,4
1 mg/L	169
	171
mean	170
Std dev.(SD)	1,4
CV%	0,8
1,5 SD	2,1
Mean +/- 1.5 SD	167,9 – 172,1
2 SD	2,8
Mean +/- 2SD	167,2 – 172,8
10 mg/L	143
	142
mean	142,5
Std dev.(SD)	0,7
C V%	0,5
1,5 SD	1,1
Mean +/- 1.5 SD	141,4 – 143,6
2 SD	1,4
Mean +/- 2SD	141,1 – 143,9

Table 11: Detection of anti-mitochondrial type M2 antibodies**Table11 a:** 1° Comparison with the ELISA method

5

Reagents
Membrane : 1082
Conjugate: 1051
Diluent buffer: 1081
Fixative :1021

10

Sample n°	FT (Ru's)	Int.	ELISA (OD 450 nm)	Int.
<i>positive</i>				
34590	135	+	1,836	+
4820	128	+	1,905	+
26416	90	+	1,567	+
18133	119	+	1,481	+
8413	121	+	1,800	+
5276	90	+	1,510	+
<i>negative</i>				
49319	161	-	0,206	-
49556	154	-	0,178	-
3494	158	-	0,064	-
2364	149	-	0,049	-
49435	164	-	0,036	-
40855	147	-	0,075	-
3494	161	-	0,049	-
2364	154	-	0,064	-
46235	156	-	0,040	-
<i>Cut-off</i>	143	-	<0,300	
Blank value	164			

Table 11b: Detection of anti-mitochondrial type M2 antibodies.

Study of the inter-assays reproducibility

5	Reagents
	Membrane : 1081 / 1082 / 1083/ 1085
	Conjugate: 1051 Diluent buffer: 1081 Fixative :1021

Positive sample	RU's	RU's	RU's	RU's
Lot membrane	1081	1082	1083	1085
5276	90	80	np	80
		89		
		90		
Mean	86			
Std Dev.	5,3			
CV %	6,2			
26416	89	106	95	82
	90			
Mean	92			
Std Dev.	8,9			
CV %	9,6			
18133	119	104	109	98
Mean	107			
Std Dev.	7,7			
CV %	7,2			

10

Negative samples	RU's	RU's	RU's
Lot membrane	1081	1082	1083
3494	161	152	167
Mean	160		
Std Dev.	7,5		
CV %	4,7		
2364	153	159	148
Mean	153		
Std Dev.	5,5		
CV %	3,6		
49435	153	140	153
Mean	149		
Std Dev.	7,5		
CV %	5,0		

Table 12: Detection of *Salmonella*.

Feasibility of quantification

Dilution factor	Counting of bacteria /ml	Reflectance units	
		a) Volumes 2 x 250 µl	b) Volume 1 X 500 µl
10-1	$5,30 \times 10^7$	92	25
10-2	$5,0 \times 10^6$	67	
10-3	$5,80 \times 10^5$	np	
10-4	$6,3 \times 10^4$	106	50
10-5	$5,6 \times 10^3$	107	
10-6	$5,9 \times 10^2$	np	
10-7	$6,5 \times 10^1$	np	
10-8	7,0	np	96
blank		170	170

Table 13: influence of the composition of the diluent buffer

Sample	Buffer 1111 (A)	Buffer 1112 (A)	Buffer 1051 (B)	Buffer 1113 (C)
	Ru's	Ru's	Ru's	Ru's
3 (1,2 mg/L)	175	171	172	182
	166	175	177	182
	167	165	174	177
	74	169	170	178
Mean	170	170	176	180
Std dev.	4,9	4,2	3,0	2,6
CV's %	2,8	2,4	1,7	1,5
15 (9,3 mg/L)	152	151	157	157
	155	162	157	161
	159	153	152	160
	165	154	160	150
Mean	158	155	157	157
Std dev.	5,6	4,8	3,3	5,0
CV's %	3,6	3,1	2,1	3,2
21 (20 mg/L)	142	137	119	123
	128	142	137	136
	133	141	141	143
	137	136	122	130
Mean	135	139	130	132
Std dev.	5,9	2,9	10,8	9,7
CV's %	4,4	2,1	8,4	7,4
36 (84 mg/L)	101	103	102	89
	103	115	108	95
	102	117	99	94
	101	112	112	101
Mean	102	112	105	95
Std dev.	0,9	6,2	5,9	4,9
CV's %	0,9	5,5	5,6	5,2
50 (268 mg/L)	80	68	71	74
	79	70	91	69
	81	74	85	71
	77	71	80	65
Mean	79	71	82	70
Std dev.	1,7	2,5	8,5	3,8
CV's %	2,2	3,5	10,3	5,4

Table 14: Correlation with another rapid method for the detection of CRP by immunoturbidimetry**Table 14a:** Correlation on negative samples

Number of samples	BIO ART conc. mg/L	ORION conc. mg/L
12	0-9	< 8
15	10	< 8
3	10-25	< 8

5

Table 14b: Correlation on low positive samples (conc. from 9 to 26 mg/L)

1. Sample n°	2. BIO ART conc. mg/L	3. ORION conc. mg/L	4. correlation
5. 5	6. 25	7. 19	8. Ok
9. 7	10. 10	11. 9	12. Ok
13. 10	14. 25	15. 16	16. Ok
17. 11	18. 25	19. 19	20. Ok
21. 13	22. 10	23. 12	24. Ok
25. 21	26. 10-25	27. 11	28. Ok
29. 22	30. 10-25	31. 26	32. Ok
33. 26	34. 25	35. 15	36. Ok
37. 30	38. 10-25	39. 13	40. Ok
41. 31	42. 25-50	43. 25	44. Ok
45. 33	46. 25	47. 10	48. Ok
49. 42	50. 10	51. 18	52. Ok

10 **Table 14c:** correlation on positive samples between 27 and 100 mg/L

Sample n°	BIO ART conc. mg/L	Orion conc. mg/L	Correlation
14	75-100	77	ok
15	25-50	27	ok
18	25-50	50	ok
40	50	44	ok

Table 14d

Sample n°	BIO ART conc. mg/L	Orion conc. mg/L
37	200	112

Table 14e: general conclusion

5

ORION ***+ results****- results**

10

BIO ART + results**17****3**

15

- results**0****27**

20

Total**17****30**

25

Sensitivity :**17/17****100%****Specificity: 27/30****90%**

Claims

1. An assay device for testing the presence of an analyte in a given sample comprising: a multilayer support whereon a first analyte-binding compound or analyte-binding complex, able to bind said analyte present in said sample, is immobilized, whereby said analyte is able to bind a second enzyme labeled analyte-binding compound or enzyme labeled analyte-binding complex forming a sandwich complex, whereby said sandwich complex is able to generate upon contact with a suitable precipitating substrate for said enzyme-label a colored deposit in a one step procedure.
2. An assay device according to claim 1, wherein said multilayer support comprises:
 - a) an upper cover layer of a water-impermeable material having at least one hole, whereby said hole at least partly exposing a test zone,
 - b) an intermediate porous layer comprising at least one insoluble porous material whereon the first analyte binding compound is able to bind in said test zone, and
 - c) a lower absorbent layer comprising at least one layer of a hydrophilic material.
3. An assay device according to claim 2 wherein the water-impermeable material is made out a plastic material adapted to the sample to be tested.
4. An assay device according to claims 2 or 3 wherein said test zone has a diameter of at least 1 mm.
5. An assay device according to claim 4 wherein said test zone is preferential 3 to 4 mm.
6. An assay device according to any of the claims 2 to 5 wherein the intermediate insoluble porous material is made from a material chosen from the group comprising nylon, nitrocellulose, cellulose, fiberglass, polysulfone, polyvinylidene difluoride, polyester or any other polymeric material whereon a biological substance may bind.
7. An assay device according to claim 6 wherein the intermediate insoluble porous material is nitrocellulose.
8. An assay device according to claims 6 and 7 wherein the intermediate insoluble porous material has pores with a diameter between 0.1 and 12 μm , and a thickness up to 2500 μm .

9. An assay device according to claim 8 wherein the intermediate insoluble porous material has pores with a diameter preferably of 0.45 μm and a thickness of 500 μm .
10. An assay device according to any of the claims 1 to 9, wherein the hydrophilic material of the device allows communication between the porous material and the absorbent layer and is AP120 or any equivalent absorbent pad.
11. An assay device according to any of the claims 1 to 10, wherein said first analyte-binding compound and said second analyte-binding compound are substances which specifically bind the analyte and are chosen from the group comprising peptides, proteins, lipids, nucleic acids and organic molecules.
12. An assay device according to claim 11 wherein said first analyte-binding compound and/or said second analyte-binding compound is preferentially an antibody which specifically binds the analyte.
13. An assay device according to claim 12 wherein said antibody is a monoclonal or polyclonal or an antibody preparation thereof.
14. An assay device according to any of the claims 1 to 13, wherein said first analyte-binding compound is directly coupled to the porous layer of said device.
15. An assay device according to any of the claims 1 to 13, wherein said first analyte-binding compound is indirectly coupled to the porous layer of said device.
16. An assay device according to claim 15 wherein the porous layer of said device is first coated with a capturing molecule which specifically binds said first analyte-binding compound followed by the coating of said analyte-binding compound.
17. An assay device according to any of the claims 1 to 16 wherein said analyte is detected indirectly by the use of a detection molecule.
18. An assay device according to claim 1-17 wherein said second analyte-binding compound or analyte-binding complex is able to bind with a detection molecule labeled with a different enzyme (E2) than the enzyme present on said second analyte-binding compound (E1),

whereby said E2 enzyme upon interaction with a precipitating substrate using a one step procedure results in a colored deposit.

19. An assay device according to claim 1-18, wherein said second analyte-binding compound is
5 not enzyme-labeled and is further bound by a detection molecule labeled with an enzyme which upon interaction with a precipitating substrate using a one step procedure results in a colored deposit.
20. An assay device according to any of claims 15-19, whereby said Indirect coupling is realized
10 via an avidin/biotin, biotin/streptavidin, antibody/antigen, antibody/hapten, receptor/ligand, sugar/lectin, complementary nucleic acid, enzyme/substrate, enzyme/cofactor, enzyme/inhibitor or Immunoglobulin/ Staphylococcal proteinA interaction.
21. An assay device according to any of the claims 1 to 20 wherein said sample can be chosen
15 from a group comprising cell fractions, serum, whole blood, urine, plasma for human or animal diagnostic testing; soil, mud, minerals, water, air for environmental testing; any food materials for food testing; or any other medium/suspension/hard material which can be used for one of these purposes.
22. An assay device according to any of the claims 1 to 21 wherein said test sample can be
20 applied undiluted or in a diluted form using a diluent buffer and for which the dilution factor is adapted to the analyte to be detected.
23. An assay device according to claim 22 wherein said dilution is chosen between $\frac{1}{2}$ up to
25 1/100.000.
24. An assay device according to claims 22 or 23 wherein said diluent buffer has a composition adapted to the analyte to be detected.
25. An assay device according to any of the claims 1 to 24 wherein said analyte is a compound
30 abnormally or normally present or absent in the sample.
26. An assay device according to claim 25 wherein said compound is selected from the group
35 comprising antigens, antibodies whereby said antigen is chosen from the group comprising any biological agent such as bacteria, viruses, molds, mycobacteria, parasites, pathogens; or

any molecule such as peptides, proteins, lipids, organic molecules and nucleic acid oligomers.

27. An assay device according to claim 26 wherein said antigen is chosen from the group comprising proteins for which the level abnormally increases in certain diseased states or abnormally increases in food material.
28. An assay device according to claim 26 wherein said antigen is chosen from the group comprising C-reactive protein (CRP), troponin, myoglobin, HCG (human chorionic gonadotrophin, rheumatoid factors, cardiolipin, centromere (kinetochore proteins), histones, Jo-1 (eponymously named, same as histidyl tRNA transferase, RNP (ribonucleoproteins eg. U1RNP)), lupus coagulant, myeloperoxidase, nucleolar auto-antigens (e.a.: PM-Scl = polymyositis-Scleroderma) (eg. U1RNP), Scl70 (same as topoisomerase 1), Sm (eponymously named as Smith antigen, same as nuclear antigen), SSA/Ro (Sjögren syndrome antigen), SSB/La (Sjögren syndrome antigen, thyroglobulin, cell surface lipoproteins, Thyroid auto-antigens, collagen, ANCA (anti-neutrophil cytoplasmic antibodies).
29. An assay device according to claim 26 wherein said antibody belong to any class of immunoglobulin comprising IgE, IgG, IgM, IgA, IgD.
30. An assay device according to claim 26 wherein said nucleic acid oligomer is chosen from the group comprising DNA, RNA, DNA/RNA hybrid or chemically analogues thereof, genetically modified or not.
31. An assay device according to any of the claims 1 to 30 whereby said enzyme-label, which is coupled to said second analyte binding compound or said detection molecule reacts in one step with a precipitating substrate and is chosen from the group comprising horse radish peroxidase (HRP), alkaline phosphatase, and dehydrogenase.
32. An assay device according to any of the claims 1 to 31 wherein said enzyme-label is covalently or non-covalently bound to the second analyte-binding compound.
33. An assay device according to any of the claims 31 or 32 wherein said enzyme label is HRP and said precipitating substrate is chosen from the group comprising TMB (tetramethylbenzidine) and AEC (3-amino-9 ethylcarbazole).

34. An assay device according to any of the claims 31 or 32, wherein said enzyme label is alkaline phosphatase and said substrate is chosen from the group comprising BCIP (5-bromo-4-chloro-3-indolyl phosphate) and BCIP-NBT (5-bromo-4-chloro-3-indolyl phosphate-nitro blue tetrazolium).
- 5
35. An assay device according to any of the claims 31 or 32 wherein said enzyme label is dehydrogenase and said substrate is chosen from the group comprising NBT (nitro blue tetrazolium).
- 10
36. An assay device according to claim 33 wherein the formed TMB precipitate is able to be fixated using a reagent chosen from the group comprising polyvinyl alcohol supplemented with diethyl sulfosuccinate and dimethyl formamide.
- 15
37. An assay device according to any of the claims 1 to 36 whereby the analysis is performed qualitatively, semi-quantitatively or quantitatively.
38. An assay device according to any of the claims 1 to 37 containing at least one test zone which may be used for standard(s) and/or positive and/or negative and/or cut-off control(s).
- 20
39. An assay device according to any of the claims 1 to 38 wherein the color deposit is observed and interpreted using a card system or using a reader.
- 25
40. A diagnostic kit for testing the presence of an analyte in a given sample comprising:
- an assay device as defined in any of the claims 1 to 39 provided with a first analyte-binding compound or analyte-binding complex,
 - a second solution comprising an enzyme-labeled second analyte-binding compound, an enzyme-labeled second analyte-binding complex or an enzyme-labeled detection molecule,
 - a third solution comprising a precipitating substrate for the enzyme linked to the second analyte-binding compound, an enzyme-labeled second analyte-binding complex or an enzyme-labeled detection molecule able to generate a colored deposit upon reaction with said enzyme,
 - optionally a color chart for the interpretation of the colored deposit, and
 - optionally an instruction leaflet.
- 30
- 35

41. A diagnostic kit according to any of the claim 40 or an assay device according to any of the claims 1 to 39 for use in testing/controlling/detecting clinical (human or animal), agricultural, environmental or food samples.
- 5 42. A diagnostic kit according to claims 40 or 41 or an assay device according to any of the claims 1 to 40 for the diagnosis and/or monitoring of treatment of diseases.
43. A diagnostic kit according to claims 40 or 41 or an assay device according to any of the claims 1 to 40 for the diagnosis and/or monitoring of treatment of auto-immune diseases
10 induced by organ or non-organ specific auto-antigens.
44. A diagnostic kit according to claim 43 wherein said non-organ specific (multisystem) auto-immune disease is chosen from the group of diseases comprising systemic lupus erythematosus (SLE) and other rheumatic diseases, scleroderma with or without Crest
15 syndrome, drug-induced lupus erythematosus (LE), polymyositis with or without scleroderma, primary Sjögren syndrome, rheumatoid arthritis, and connective tissue diseases.
45. A diagnostic kit according to claim 43 wherein said organ specific auto-immune disease is chosen from the group of diseases comprising Addison's disease, auto-immune haemolytic
20 anemia, chronic active hepatitis, coeliac disease, Goodpasture's syndrome, Grave's thyrotoxicosis, Hashimoto's thyroiditis, Idiopathic thrombocytopenic purpura, Juvenile-onset diabetes, late onset diabetes, lens induced uveitis, some male infertility, multiple sclerosis, myasthenia gravis, primary biliary cirrhosis, pernicious anemia, primary myxoedema, sympathetic ophtalmia, ulcerative colitis, vasculitides, pemphigoid and Wegener's
25 granulomatosis.
46. A diagnostic kit according to any of the claims 40 or 41 or an assay device according to any of the claims 1 to 39 for the diagnosis and/or monitoring of treatment of infectious diseases
30 included by viruses, bacteria, molds, mycobacteria or parasites.
47. A diagnostic kit according to any of the claims 40 or 41 or an assay device according to any of the claims 1 to 39 for the diagnosis and/or monitoring of treatment of allergic diseases or
Intolerance manifestations induced by numerous allergens from grasses, weeds, molds, foods, trees, epidermals and dust.
35

48. A diagnostic kit according to any of the claims 40 or 41 or an assay device according to any of the claims 1 to 39 for use in the testing of cardiac and/or inflammatory markers.
49. A diagnostic kit according to claim 48 wherein said cardiac markers are chosen from the group comprising of myoglobin, creatine kinase and troponin and inflammatory markers are chosen from the group comprising C-reactive protein and Interleukins.
50. A diagnostic kit according to any of the claims 40 or 41 or an assay device according to any of the claims 1 to 39 for use in testing of bacteria and/or toxins.
51. A diagnostic kit according to any of the claims 40 or 41 or an assay device according to any of the claims 1 to 39 for use in testing of tumor antigens.
52. A diagnostic kit according to any of the claims 40 or 41 or an assay device according to any of the claims 1 to 39 for use in testing of drugs of abuse molecules.
53. A method for the detection of an analyte present in a test sample comprising the use of a diagnostic assay device according to any of claims 1 to 39.
54. A method according to claim 53 wherein the addition of the first analyte-binding compound, sample, second analyte-binding compound, substrate solution and fixation solution are performed subsequently, one after the other.
55. A method according to claim 54 wherein the addition of the first analyte-binding compound, sample, second analyte-binding compound, substrate solution and fixation solution are not all performed subsequently, and wherein some of these may be premixed in advance before applying them onto the device.
56. Use of a fixative solution in an assay device according to any of the claims 1 to 39 or as part of a diagnostic kit according to claims 40 to 52.
57. A method for coating a porous layer in an assay device as described in any of the claims 1 to 39 comprising the following steps:
- cutting membranes into strips,
 - immersing said strips into an application buffer and the capturing agent or first analyte-binding compound,

- Incubating the membrane,
 - Immersing the membrane in a blocking agent containing 0.2 to 10% blocking agent, whereby the blocking agent may be BSA or any other agent known to block free sites on membranes,
 - 5 - Incubating the membranes
 - drying strips, and
 - when storage is needed, packaging of the strips are packaged and stored;
- wherein the coating buffer features preferentially very low salinity and basic pH (9.1+/-2) and the analyte-binding compound is present in excess.
- 10 58. A method for coating a porous layer in a assay device as described in any of the claims 1 to 39 comprising following steps:
- cutting membranes into strips of preferably 0.8 cm wide,
 - immersing said strips into a bath brought at RT containing the application buffer and the capturing molecule or the first analyte-binding compound,
 - 15 - incubating the membrane for 3 hours at RT under gentle agitation,
 - immersing the membrane in a blocking agent containing 1% BSA,
 - incubating during 3 hours at RT under gentle agitation,
 - drying strips at 37°C in an incubator for 1 hour to overnight, and,
 - 20 - when storage is needed, packaging of the strips in order to protect membranes from humidity and stored at RT;
- wherein the coating (application) buffer features preferentially very low salinity and basic pH (9.1+/-0.1), the analyte-binding compound is present in an excess.
- 25 59. A method using a device as described in claims 1 to 39 comprising following steps:
- dilute sample 1/2 to 100.000 into the diluent buffer, whereby the diluent buffer is a Tris buffer or of another composition of low salinity and containing 1 to 5 % BSA, or use an undiluted sample,
 - applying 15 µl or one drop of diluted sample on the membrane,
 - 30 - allowing the sample to soak for 1 minute at least (range : 30" up to 1'30") preferentially 45",
 - applying 25 µl or one drop of conjugate (anti-CRP coupled to HRP) and allow to soak,
 - applying 25 µl or one drop of precipitating TMB and allow to soak,
 - apply 25 µl or one drop of fixative solution and allow to soak,
 - 35 - waiting for 2 minutes before reading the result and read within 30 minutes, and,

- covering the colored spot with a scotch band (type 3M) when a long-term storage of the result is necessary.

INTERNATIONAL SEARCH REPORT

In ☐ national Application No
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According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ

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☐ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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INTERNATIONAL SEARCH REPORT

Information on patent family members

In International Application No

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